Tomonobu Kusano · Hideyuki Suzuki
*Editors*

# Polyamines

## A Universal Molecular Nexus for Growth, Survival, and Specialized Metabolism

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# Preface

The first description of polyamines (PAs) was presented in 1678 by Antonie van Leeuwenhoek, known as the "Father of Microbiology". With his improved microscope, he discovered the presence of crystalline substances in human semen. In 1924, some 250 years after that initial finding, such crystals were identified as spermine phosphate by Otto Rosenheim. Since then, many researchers have devoted themselves to clarifying the multiple functions of PA molecules that are present in almost all living organisms, and great progress has been made in PA research over the last 90 years. Here, we summarize our current understanding about PAs in bacteria, Archaea, and eukaryotes.

This book covers almost all the basic information about PA biosynthesis, catabolism, and regulatory mechanisms that maintain cellular PA levels. With the availability of genome sequence information from various organisms, it is now possible to extensively discuss the evolution of the PA metabolic genes. The effects of PAs on growth and survival, as well as their effects on ion channels, are also covered. Prokaryotic transporters are summarized, and recently identified plant PA transporters are also described. PA molecules are known to be precursors of secondary metabolites, such as plant alkaloids and toxins originated from spiders and wasps, and the functions and synthetic pathways of these specialized metabolites are also introduced. Because how PAs contribute to longevity has recently become a hot topic, some of the new findings in the animal and plant fields are overviewed. Also, a positive link between cancer progression and PA contents is known, and thus the use of PA metabolite(s) as diagnostic markers in the initial stages of cancer is introduced. Lastly, a novel approach to use the PA pathway of a parasite as a drug target in African sleeping sickness is described.

This book therefore encompasses a broad range of PA research fields, from basic to the applied sciences, with each chapter not only describing basic information but also the most up-to-date knowledge currently available.

We hope readers will find this volume useful and interesting.

Sendai, Japan — Tomonobu Kusano
Kyoto, Japan — Hideyuki Suzuki

# Contents

# Part I
# Biosynthesis of Polyamines

# Chapter 1
# Biosynthesis of Polyamines in Eukaryotes, Archaea, and Bacteria

**Anthony J. Michael**

**Abstract** As with all metabolic pathways, not only has polyamine biosynthesis been subject to divergent and convergent evolution and horizontal gene transfer, but many pathogenic and commensal organisms have abandoned the task altogether and instead obtain polyamines from the environment. Reflecting primary metabolism in general, polyamine biosynthesis is more diverse in Bacteria than it is in eukaryotes and Archaea. Each of the three physiologically relevant triamines, that is, spermidine, *sym*-homospermidine, and *sym*-norspermidine, can be synthesized by at least two distinct, evolutionarily independent pathways. Synthesis of tetraamines has evolved independently in Bacteria, Archaea, plants, yeasts, and animals. Because of the relative ease of genomic sequencing and the ever-increasing number of complete genome sequences available, it will now be easier to determine which polyamines are likely to be present in an organism by using genomic rather than chemical analysis. The following chapter is a guide to the biosynthetic diversity of polyamine formation and the evolutionary mechanisms generating that diversity.

**Keywords** Agmatine • Archaea • Bacteria • Carboxyspermidine • Eukaryote • Homospermidine • Norspermidine • Polyamine • Spermidine • Thermospermine

## 1.1 Introduction

Eukaryotic and archaeal cells must synthesize or take up spermidine because it has an essential role in posttranslational modification of the translation factor IF5A, known as hypusination (Park et al. 2010). In baker's yeast, hypusinated eIF5A has been shown to stimulate the peptidyl transferase activity of the ribosome and to be essential for translation of mRNAs encoding polyproline tracts (Gutierrez et al. 2013). The analogous bacterial translation factor EF-P is also required for rapid translation of mRNAs containing polyproline tracts (Doerfel et al. 2013; Ude et al. 2013). In contrast to the spermidine-dependent hypusine modification of eIF5A, the bacterial EF-P

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protein is modified by the addition of (R)-β-lysine in a posttranslational modification known as lysinylation (Yanagisawa et al. 2010; Roy et al. 2011). Therefore, in eukaryotes and Archaea, in contrast to Bacteria, cell growth and proliferation are absolutely dependent on spermidine biosynthesis or its acquisition through uptake transport.

The evolutionary event that gave rise to the hypusine modification of eIF5A in the common ancestor of Archaea and eukaryotes effectively locked life to spermidine biosynthesis or uptake. Deoxyhypusine synthase, which transfers the aminobutyl group of spermidine to eIF5A, is an essential gene in single-celled eukaryotes such as *Leishmania donovani* (Chawla et al. 2010), *Trypanosoma brucei* (Nguyen et al. 2013), and baker's yeast (Park et al. 1998), and also in the mouse (Nishimura et al. 2012). Bacterial life is not constrained by the need for spermidine in the modification of an essential translation factor, and bacteria may synthesize a diverse range of polyamines, including the triamines *sym*-norspermidine, spermidine, and *sym*-homospermidine, or none at all (Hamana and Matsuzaki 1992). There may be other essential roles of spermidine in eukaryotic life, but it is difficult to separate the role of spermidine in eIF5A modification from its role in other cellular processes, although it has been shown that polyamines may affect translation initiation in a manner different from the effect of eIF5A (Landau et al. 2010). In most animals (metazoa), in *Saccharomycotina* yeasts, and in flowering plants, spermidine is a precursor for spermine biosynthesis and for thermospermine biosynthesis in plants (Pegg and Michael 2010).

In contrast to the essential requirement for spermidine in eukaryotic and archaeal life, the role of polyamines in bacteria is less clear. Spermidine is essential for planktonic growth of the gram-negative γ-proteobacterium *Pseudomonas aeruginosa* PAO1 (Nakada and Itoh 2003) and the ε-proteobacterium *Campylobacter jejuni* (Hanfrey et al. 2011), whereas the gram-positive firmicute *Bacillus subtilis* does not require polyamines for normal planktonic growth (Burrell et al. 2010). In other γ-proteobacteria, polyamine depletion reduces planktonic growth rate by approximately 40 %, for example, *Yersinia pestis* (Patel et al. 2006), *Vibrio cholerae* (Lee et al. 2009), *Salmonella typhimurium* (Green et al. 2011), and *Escherichia coli* (Chattopadhyay et al. 2009). Thus, there may not be a conserved core role of polyamines in bacterial physiology but rather a diverse range of functions dependent on ecological and physiological context.

## 1.2 Polyamine Biosynthesis

The evolutionary processes shaping the formation of biosynthetic pathways include gene duplication, gene loss, gene fusion, and horizontal and endosymbiotic gene transfer. Complete or partial loss of polyamine biosynthesis is a prominent characteristic of single-celled parasites, which have subsequently become dependent on polyamine uptake. Examples include the loss of ornithine decarboxylase (ODC) in *Trypanosoma brucei*, *T. conglese*, *T. vivax*, and *T. cruzi*, followed by the reacquisition of ODC by horizontal gene transfer from a vertebrate source in the African

trypanosome lineage but not in *T. cruzi* (Steglich and Schaeffer 2006). The related *Leishmania* parasites did not lose the ODC gene. Genomic inspection indicates that spermidine biosynthesis has also been lost in *Giardia*, *Trichomonas*, *Toxoplasma*, *Cryptosporidium*, *Entamoeba*, and *Microsporidia* (unpublished results). Gene duplication and subsequent neo-functionalization of the additional gene copy has given rise to spermine synthase from spermidine synthase in *Saccharomycotina* yeasts and flowering plants (Pegg and Michael 2010), as well as the evolution of antizyme inhibitor from ODC (Murakami et al. 1996). Gene fusion of two bacterial *S*-adenosylmethionine decarboxylase (AdoMetDC) open reading frames gave rise to the eukaryotic AdoMetDC (Toms et al. 2004), and gene fusion of a bacterial AdoMetDC to the N-terminus of bacterial spermidine synthase, followed by loss of catalytic function of the AdoMetDC domain, gave rise to the metazoan spermine synthase (Wu et al. 2008). Plant-specific acquisition of the genes for arginine decarboxylase (ADC), agmatine iminohydrolase (AIH), and *N*-carbamoylputrescine amidohydrolase (NCPAH), which together produce putrescine from arginine, is an example of endosymbiotic gene transfer from the cyanobacterial progenitor of the chloroplast to the host nuclear genome (Illingworth et al. 2003). The following sections briefly describe polyamine biosynthesis in eukaryotes, Archaea, and Bacteria.

### *1.2.1 Eukaryotes*

Production of spermidine from putrescine is the most conserved feature of polyamine biosynthesis in eukaryotic cells. However, there are alternative putrescine biosynthetic routes, and tetraamine biosynthesis has evolved independently several times. Except for plants, all other eukaryotes synthesize putrescine from ornithine using ODC (Fig. 1.1), which is a head-to-tail homodimer with the active site formed across the dimer interface (Pegg 2006). The form of ODC found in eukaryotes was thought to be specific to eukaryotes; however, orthologues from the same structural class are also found in bacteria, especially α-proteobacteria (Lee et al. 2007). This finding may explain the evolutionary provenance of the eukaryotic ODC, because the *Rickettsiales* order of the α-proteobacteria is thought to be the origin of the mitochondrion (Andersson et al. 2003). Paradoxically, extant members of the *Rickettsiales*, which are intracellular pathogens, have lost the genes for polyamine biosynthesis. An α-proteobacterial ODC gene in the mitochondrial progenitor could have been transferred by endosymbiotic gene transfer to the host nucleus. Regulation of ODC activity by the extraordinary antizyme–antizyme inhibitor system will be described elsewhere in this book.

The case for endosymbiotic origin of the genes for putrescine biosynthesis from arginine in plants (ADC, AIH, and NCPAH) via the cyanobacterial progenitor of the chloroplast is very strong (Illingworth et al. 2003). Most plants therefore have two pathways for putrescine biosynthesis (Fig. 1.1): from ornithine, and from arginine (Fuell et al. 2010). Some plants, including *Arabidopsis thaliana* and moss, have lost the ODC pathway to putrescine, whereas some single-celled green algae have lost

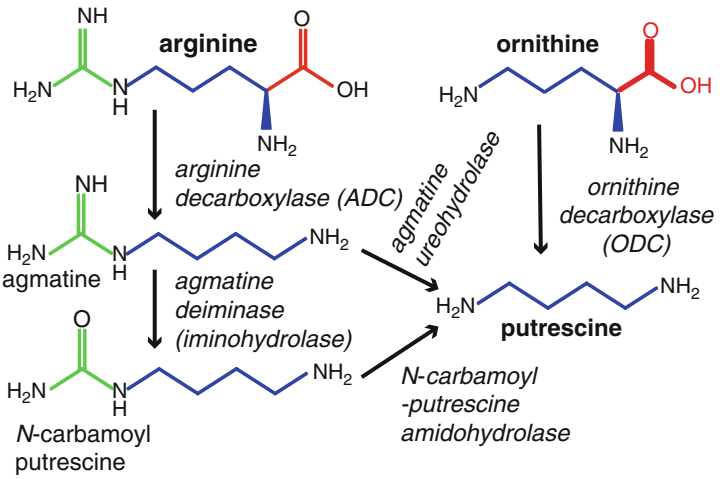


**Fig. 1.1** Putrescine biosynthetic pathways

the ADC pathway (Fuell et al. 2010). In some single-celled eukaryotes, ODC is present in the absence of the rest of the spermidine pathway, for example, *Trichomonas vaginalis* (Yarlett et al. 2000).

The pathway for conversion of putrescine to spermidine is highly conserved in eukaryotes (Fig. 1.2). Spermidine synthase (SpdSyn) is an aminopropyl transferase found in all three domains of life and transfers an aminopropyl group from decarboxylated *S*-adenosylmethionine (dcAdoMet) to putrescine to form spermidine (Wu et al. 2007). Decarboxylation of AdoMet is performed by AdoMetDC, a pyruvoyl-dependent enzyme, which was likely present in the last eukaryotic common ancestor. The pyruvoyl group is generated from an internal serine residue exposed by an autocatalytic self-cleavage reaction that is stimulated by putrescine in mammalian cells (Pegg 2009). Translation of AdoMetDC mRNA is regulated by small upstream open reading frames encoding inhibitory peptides in both mammalian and plant cells (Raney et al. 2002; Hanfrey et al. 2005; Ivanov et al. 2010). In *Plasmodium falciparum*, the AdoMetDC open reading frame is fused to the N-terminus of ODC to form a bifunctional enzyme (Muller et al. 2000); a similar fusion of AdoMetDC and ODC is found in single-celled green algal *Micromonas* species (Green et al. 2011).

Synthesis of tetraamines has evolved independently several times in eukaryotes. Spermine is formed by the transfer of an aminopropyl group from dcAdoMet to the aminobutyl side of spermidine to form a symmetrical tetraamine (Fig. 1.2). In humans, the spermine synthase (SpmSyn) X-ray crystal structure revealed that the enzyme is a fusion between a noncatalytic bacterial AdoMetDC-like domain at the N-terminus and a spermidine synthase-like domain (Wu et al. 2008). The N-terminal AdoMetDC-like domain is essential for dimer formation and enzymatic activity although it is not catalytic and no autocatalytic self-processing occurs. Human-type SpmSyn is found in almost all animals (Metazoa) except nematodes and in some choanoflagellates, which are the closest single-celled relatives of metazoa (Pegg and Michael 2010). Fusions of a functional AdoMetDC and a

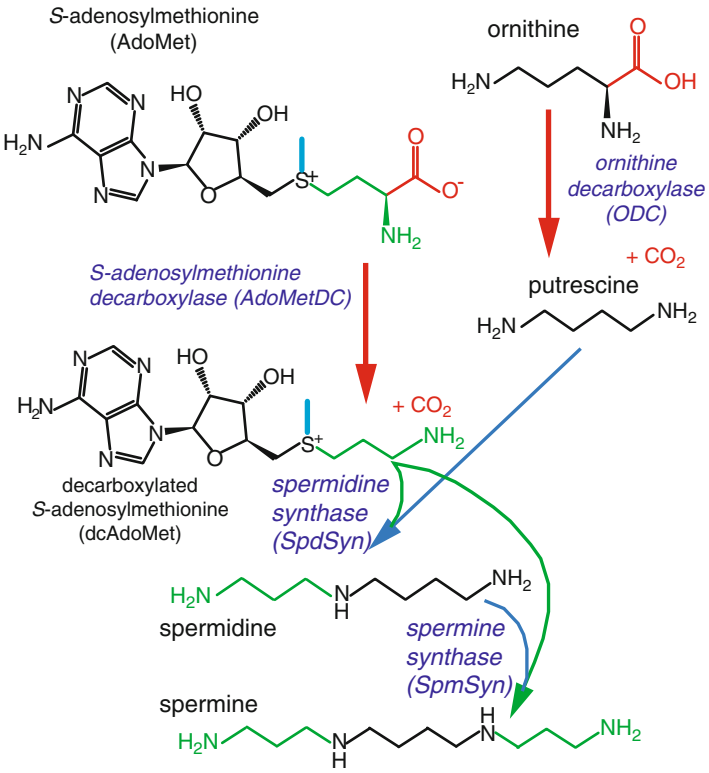


**Fig. 1.2** Spermidine and spermine biosynthesis in eukaryotes

functional aminopropyl transferase are found in diverse bacterial phyla (Green et al. 2011), and it is likely that the origin of the human-like SpmSyn was the acquisition of such a bacterial gene fusion by horizontal gene transfer in the single-celled common ancestors of animals. In contrast, the yeast SpmSyn represents a relatively recent gene duplication of the SpdSyn gene and subsequent evolution of an altered substrate specificity so that spermidine rather than putrescine is recognized. The yeast-type SpmSyn is found in only a small group of yeast species, the *Saccharomycotina* yeasts, which includes *Candida albicans*, whereas the rest of the fungi do not possess a SpmSyn gene and do not accumulate spermine (Pegg and Michael 2010). Similarly, the SpmSyn of flowering plants evolved independently by gene duplication of SpdSyn and is not found in nonflowering plants (Pegg and Michael 2010).

In contrast to the relatively recent evolution of spermine biosynthesis in plants, synthesis of the unsymmetrical tetraamine thermospermine is found throughout the plant lineage (Pegg and Michael 2010). Thermospermine is synthesized by an aminopropyl transferase, thermospermine synthase (TspmSyn), that transfers an aminopropyl group from dcAdoMet to the aminopropyl side of Spd to form an unsymmetrical tetraamine. Not only are homologues of TspmSyn found in the plant

lineage, but they are also present in diatoms and related phyla, probably as a result of secondary endosymbiosis events and subsequent transfer of the TspmSyn gene from the red algal endosymbiont to the host nucleus (Pegg and Michael 2010). In the flowering plant *Arabidopsis thaliana*, genetic depletion of thermospermine results in a severe stem growth defect (Hanzawa et al. 2000; Knott et al. 2007), whereas spermine is dispensable for normal growth and development (Imai et al. 2004). However, Spm is required for resistance to drought stress (Yamaguchi et al. 2007).

### *1.2.2 Archaea*

The general configuration of polyamine biosynthesis in Archaea consists of putrescine production by ADC and agmatinase (agmatine ureohydrolase, AUH), and spermidine production by AdoMetDC and SpdSyn. However, differing from plants, which have a pyridoxal 5′-phosphate-dependent ADC, the ADC of Archaea is a small pyruvoyl-dependent enzyme. The euryarchaeote *Methanocaldococcus jannaschii* ADC exhibits nonhydrolytic self-cleavage at a serine–serine peptide bond in the proenzyme to form a 5-kDa β-subunit and a 12-kDa α-subunit (Graham et al. 2002). Similarly to AdoMetDC, the pyruvoyl group formed after autocatalytic cleavage is found at the N-terminus of the α-subunit. In contrast to the euryarchaeotal ADC, the crenarchaeon *Sulfolobus solfataricus* possesses an ADC that is homologous to the archaeal AdoMetDC and has arisen by gene duplication of AdoMetDC early on in crenarchaeal evolution (Giles and Graham 2008). Change of substrate specificity from AdoMet to arginine appears to be determined by the N-terminal domain. The AUH of *Pyrococcus horikoshii* is similar to bacterial orthologues and is dependent on $Co^{2+}$, $Ca^{2+}$, or $Mn^{2+}$ (Goda et al. 2005); however, the AUH of *M. jannaschii* is $Fe^{2+}$ dependent (Miller et al. 2012). Although the AdoMetDC of Archaea (Kim et al. 2000) is similar to the typical bacterial AdoMetDC exemplified by the *Thermotoga martima* enzyme (Toms et al. 2004), the aminopropyl transferase of both Crenarchaeota and Euryarchaeota appears to be an agmatine aminopropyl transferase producing aminopropyl agmatine (Cacciapuoti et al. 2007; Morimoto et al. 2010), suggesting that the archaeal AUH enzymes that have been characterized may be aminopropyl agmatine ureohydrolases rather than agmatine ureohydrolases. Production of long-chain and branched polyamines from thermophilic Archaea is covered elsewhere in this book. An important gap in our knowledge of polyamine biosynthesis in Archaea is the formation of *sym*-norspermidine. Although it has been shown that the spermidine biosynthetic pathway aminopropyl transferase of *Sulfolobus solfataricus* can use 1,3-diaminopropane as a substrate to produce norspermidine, we still do not know how 1,3-diaminopropane is produced in Archaea outside the Halobacteria. Within the Halobacteria, 2,4-diaminobutyrate aminotransferase (DABA AT) and 2,4-diaminobutyrate decarboxylase (DABA DC), which together synthesize 1,3-diaminopropane from aspartate β-semialdehyde (Lee et al. 2009), are present in gene clusters, probably expressing schizokinin-like siderophore biosynthetic enzymes (Burrell et al. 2012) that have been acquired from bacteria by horizontal gene transfer. One possible source of 1,3-diaminopropane in

Archaea outside the Halobacteria could be the oxidation of spermidine to give 1,3-diaminopropane and 4-aminobutyraldehyde, although such an activity has not been reported in Archaea.

### *1.2.3 Bacteria*

Bacteria vary in the types of polyamine they produce, and they can use different biosynthetic pathways to produce the same polyamine (Fig. 1.3), and the same pathway to produce different polyamines. More prominently than in eukaryotes and Archaea, polyamine biosynthesis in bacteria is highly modular, with conspicuous horizontal transfer of not only biosynthetic modules via transfer of operons but also transfer of individual genes from within modules. As a consequence, polyamine biosynthetic pathways are configured in multiple different ways in different bacteria. Usually, bacteria produce a diamine and triamine; however, some bacteria produce longer-chain polyamines, some produce only diamines, and others, especially commensals and pathogens, do not produce any polyamines (Hamana and Matsuzaki 1992). Synthesis of spermidine is phylogenetically more widely distributed than the synthesis of *sym*-homospermidine, whereas *sym*-norspermidine synthesis has a much narrower distribution, being confined mainly to the *Vibrionales* in the γ-Proteobacteria and in some hyperthermophilic bacteria. Known bacterial pathways for the production of spermidine and *sym*-homospermidine are shown in Fig. 1.3. Synthesis of *sym*-norspermidine has been described completely only for *Vibrio cholerae*, but individual enzymes for its biosynthesis have been described in other γ-Proteobacteria (Lee et al. 2009). Similar enzymes produce *sym-norspermidine* from 1,3-diaminopropane in species such as *V. cholerae*, and spermidine from putrescine in considerably more species, that is, carboxy(nor)spermidine dehydrogenase (CANSDH/CASDH) and carboxy(nor)spermidine decarboxylase (CANSDC/CASDC). In *V. cholerae*, CANSDH and CANSDC synthesize s*ym*-norspermidine from 1,3-diaminopropane, although the same enzymes can synthesize spermidine from putrescine in the same strain (Lee et al. 2009). Similarly, CASDH and CASDC synthesize spermidine from putrescine in the ε-proteobacterium *Campylobacter jejuni*, but this species does not synthesize 1,3-diaminopropane (Hanfrey et al. 2011). No X-ray crystal structure is available for CANSDH; however, it does exhibit some homology with the homospermidine synthase enzyme (Shaw et al. 2010). The crystal structure of *C. jejuni* CASDC shows that it is closely related to *meso*-diaminopimelate decarboxylase (the last step in lysine biosynthesis), biosynthetic ADC and ODC of the alanine racemase-fold, bifunctional ODC/lysine decarboxylase, and eukaryotic antizyme inhibitor (Deng et al. 2010).

Spermidine is also synthesized from putrescine by the well-known AdoMetDC/SpdSyn route in many bacteria, and this pathway is entirely distinct from the CASDH/CASDC pathway. Three known classes of AdoMetDC are found in bacteria: a $Mg^{2+}$-dependent, class IA form typified by the *Escherichia coli* AdoMetDC, a $Mg^{2+}$-independent class IB form typical of *Bacillus subtilis* and *Thermotoga maritima* (Toms et al. 2004), and a class II form, regarded as the eukaryotic form but found

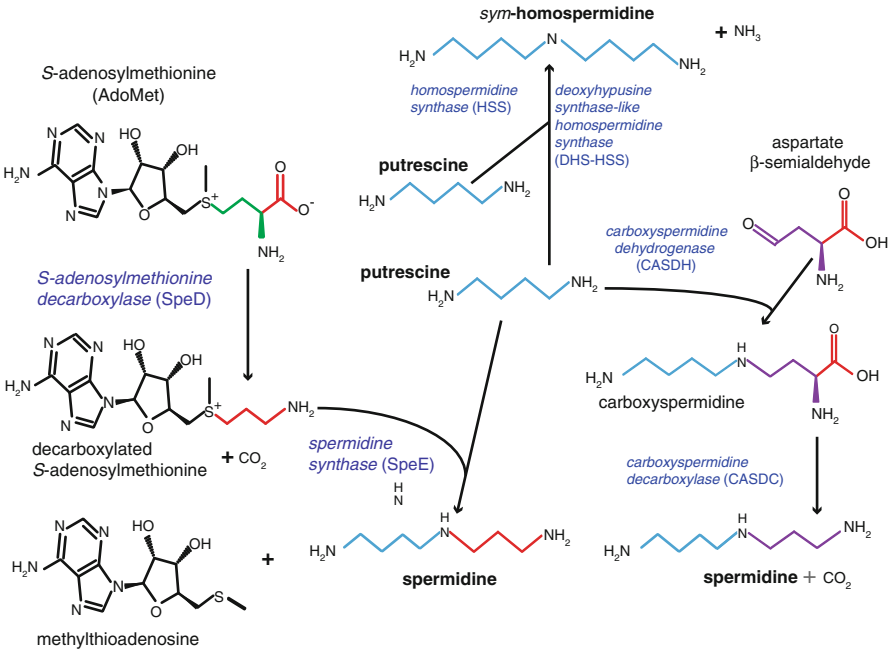


**Fig. 1.3** Spermidine and *sym*-homospermidine biosynthetic pathways in bacteria

also in many *Shewanella* species (e.g., YP_563282 from *Shewanella denitrificans* OS217), where it was probably acquired once by horizontal gene transfer from a eukaryotic source followed by vertical inheritance within the *Shewanella* genus (unpublished observations). Although most aminopropyl transferases in bacteria are likely to be spermidine synthases, some species such as *Thermus thermophilus* possess an agmatine aminopropyl transferase (Ohnuma et al. 2011). The long-chain polyamines produced by *T. thermophilus* are discussed in another chapter. Spermine is found in a surprisingly wide group of bacteria, but no specific spermine synthase has been reported (Pegg and Michael 2010). Often the AdoMetDC and SpdSyn genes are found as an operon/gene cluster, which has given rise episodically to functional AdoMetDC–aminopropyl transferase gene fusions in a phylogenetically wide range of bacteria (Green et al. 2011). Synthesis of polyamines by the AdoMetDC/SpdSyn route is expensive because of the consumption of AdoMet. A methionine salvage pathway operates in many Bacteria, Archaea, and eukaryotes to retrieve the methionine component of the SpdSyn co-product 5′-methylthioadenosine (Albers 2009). It is possible that the selection of aspartate β-semialdehyde over AdoMet for spermidine biosynthesis in many bacteria is influenced by the metabolic cost of AdoMet consumption.

An important fact for understanding the role of triamine polyamines in bacterial physiology is that many bacteria from diverse phyla produce only *sym-homospermidine* and not spermidine (Hamana and Matsuzaki 1992; Shaw et al. 2010). Therefore, any conserved role of triamines in bacteria cannot be dependent

on their exact structure, that is, symmetry and length. The corollary of this argument is that there may be no conserved role of polyamines in Bacteria, even in related species. Two pathways are present for *sym*-homospermidine synthesis from putrescine in Bacteria (Fig. 1.3): the enzyme homospermidine synthase (Tholl et al. 1996) is found predominantly in the α-Proteobacteria; and a deoxyhypusine synthase-like homospermidine synthase is found in a more diverse range of phyla (Shaw et al. 2010). Support for the role of the deoxyhypusine synthase-like enzyme in bacterial *sym*-homospermidine biosynthesis remains correlative.

The role of diamines in bacterial physiology is wider than simply to provide the precursor for *sym*-norspermidine, spermidine, and *sym*-homospermidine biosynthesis. Many iron-binding siderophore molecules are based on 1,3-diaminopropane, putrescine, or cadaverine structural backbones, such as schizokinen, acinetoferrin, putrebactin, rhizoferrin, arthrobactin, and desferroxiamines (Burrell et al. 2012). The diamines provide only a structural role in these molecules and do not participate directly in iron binding. Particularly in the case of the 1,3-diaminopropane- and cadaverine-based siderophores, the diamine biosynthetic genes are located within the siderophore biosynthetic gene cluster. Synthesis of 1,3-diaminopropane by DABA AT and DABA DC as a precursor to *sym*-norspermidine formation is limited mainly to the *Vibrionales*, where the two genes are fused to produce a fusion protein. Some species such as the γ-proteobacterium *Acinetobacter baumannii* synthesize 1,3-diaminopropane as their sole polyamine, and in the case of *A. baumannii*, synthesis of 1,3-diaminopropane is essential for surface-associated motility, which is a common trait of clinical isolates (Skiebe et al. 2012). Two molecules of 1,3-diaminopropane are present in the *A. baumannii* siderophore acinetoferrin (Okujo et al. 1994). Cadaverine is not a precursor for triamine synthesis except in a few rare cases, and it is debatable whether *N*-aminopropyl cadaverine is physiologically relevant. The lysine decarboxylase that forms cadaverine from lysine has evolved independently three times from different protein folds: once from the alanine racemase-fold (Lee et al. 2007), and twice from the aspartate aminotransferase-fold (Kanjee et al. 2011; Burrell et al. 2012). Thus, lysine decarboxylation has evolved by both convergent and pseudoconvergent evolution, that is, from different protein folds, and independently within the same protein fold.

Putrescine is the predominant precursor for triamine biosynthesis in bacteria and can be synthesized directly from ornithine or indirectly from arginine (Fig. 1.1). There are two known forms of ODC, one derived from the aspartate aminotransferase fold, exemplified by the biosynthetic ODC (SpeC) of *E. coli*; and another form homologous to the eukaryotic ODC, derived from the alanine racemase fold (Lee et al. 2007), which includes bifunctional ODC/lysine decarboxylase enzymes. In bacteria, ADC proteins can be found from at least three different folds (Burrell et al. 2010), revealing the convergent evolutionary pressure for arginine decarboxylation and polyamine biosynthesis. The product of ADC, agmatine, can be converted directly into putrescine via the activity of AUH (agmatinase), which is homologous to arginase (Ahn et al. 2004). Alternatively, agmatine deiminase and NCPAH produce putrescine from agmatine via *N*-carbamoylputrescine (Nakada and Itoh 2003), as found also in plant putrescine biosynthesis (Fig. 1.1).

## 1.3 Future Perspectives

Primary polyamine biosynthesis is not well understood in several phyla of Bacteria, most notably the Actinobacteria. An interesting area where the role for polyamine biosynthesis is not understood is in bacteriophages and viruses. In some viruses, such as the chloroviruses, an entire pathway for *sym*-homospermidine is present (Baumann et al. 2007), whereas in some bacteriophages individual genes such as functional homospermidine synthase can be found (Shaw et al. 2010). A nontrivial task for understanding the roles of polyamines in cellular physiology in the three domains of life, and also in viral and phage biology, will be to construct an atlas of polyamine biosynthetic pathways so that any new genome sequence can be easily interrogated to determine how polyamines are made by that organism. This work will be a prelude to the task of determining what polyamines do.

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# Chapter 2
# Long-Chain and Branched Polyamines in Thermophilic Microbes

**Wakao Fukuda, Ryota Hidese, and Shinsuke Fujiwara**

**Abstract** Long-chain and/or branched polyamines such as $N^4$-aminopropylspermidine [3(3)4] (abbreviation for the number of methylene $CH_2$ chain units between $NH_2$, NH, N, or $N^+$), $N^4$-bis(aminopropyl)spermidine [3(3)(3)4], and tetrakis(3-aminopropyl)ammonium [3(3)(3)3] are polycations of biotic origin that are only found in thermophiles. The thermophilic bacterium *Thermus thermophilus* and the hyperthermophilic archaeon *Thermococcus kodakarensis* synthesize a polyamine, spermidine, via conversion of arginine to agmatine (a step catalyzed by arginine decarboxylase), aminopropylation of agmatine to $N^1$-aminopropylagmatine (catalyzed by aminopropyl transferase), and hydrolysis of $N^1$-aminopropylagmatine to spermidine by $N^1$-aminopropylagmatine ureohydrolase. It is noteworthy that thermophiles synthesize spermidine without producing putrescine as an intermediate. Spermidine can be modified to produce further polyamides such as $N^4$-aminopropylspermidine [3(3)4] and then $N^4$-bis(aminopropyl)spermidine by an enzyme coded by the TK1691 gene in *T. kodakarensis*. TK1691 and its orthologues are found in (hyper)thermophilic Archaea and Bacteria, but not in mesophilic Bacteria. TK1691 is a recently characterized aminopropyl transferase involved in the synthesis of branched polyamines, which are essential for the stabilization and structural protection of nucleic acids and which enhance polypeptide synthesis at high temperature.

**Keywords** Agmatine • Aminopropyl transferase • Archaea • Branched-chain polyamine • Thermophile

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## 2.1 Introduction

Thermophilic microorganisms grow optimally above 45 °C and therefore inhabit high-temperature environments on the Earth such as hot springs, terrestrial solfatara, deep-sea hydrothermal vents, and composting organic matter. Those that grow optimally above 80 °C, known as hyperthermophiles (Stetter 1996), are all members of Archaea or Bacteria domains, which are positioned near the root of the phylogenetic tree (Fig. 2.1). This staging has led to speculation that the most ancient life forms were hyperthermophiles, although conclusive evidence to support this hypothesis is still lacking. Thermophiles are associated with specific molecular and metabolic traits such as (thermo)stable proteins (including chaperones) and physiologically active substances, as well as unique biosynthetic and other biochemical pathways that enable cellular function at high temperatures (Sato and Atomi 2011). Hyperthermophiles also produce a number of long-chain and branched polyamines.

## 2.2 Importance of Long-Chain and Branched Polyamines in Thermophilic Species

Polyamines have two or more primary amines, are polycationic, and are found in various organisms. Those such as putrescine, spermidine, and spermine are common to the cells of many phylogenetic groups. By contrast, polyamines such as

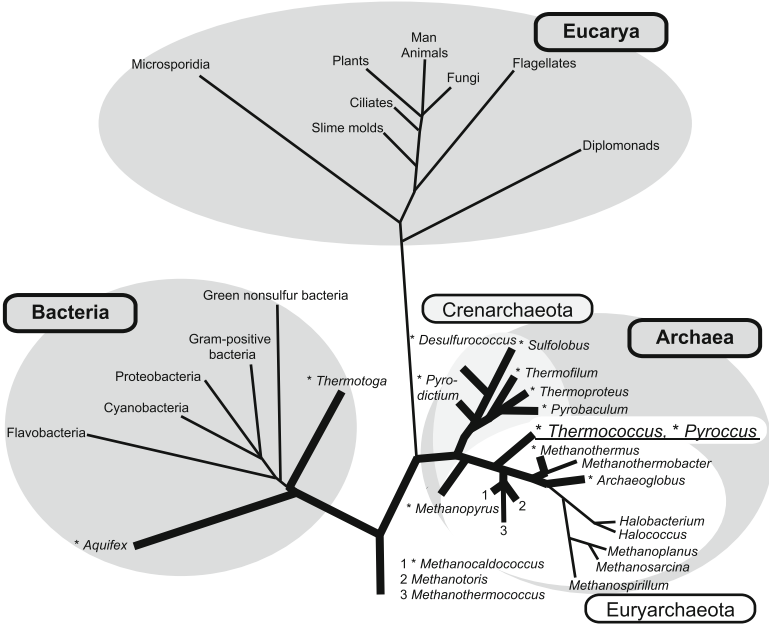


**Fig. 2.1** Phylogenetic tree based on the sequences of 16S (18S) rRNA genes. *Asterisks* indicate hyperthermophiles. *Bold lines* indicate speculated evolution routes for hyperthermophiles

cadaverine, norspermidine, homospermidine, and norspermine are confined to a small number of microbial taxa (Hamana and Matsuzaki 1985, 1992). Moreover, (hyper)thermophilic Archaea and Bacteria possess long-chain and branched-chain polyamines, which are especially important for cell growth of thermophiles at high temperature (see also Chap. 12). Quaternary branched penta-amines such as $N^4$-bis (aminopropyl)spermidine [3(3)(3)4] and isomers of $N^4$-bis(aminopropyl)spermidine, $N^4$-aminopropylspermine [3(3)43], and $N^4$-aminopropylspermidine [3(3)4] have been identified in various thermophiles. Long-chain and branched polyamines stabilize and protect nucleic acids and activate cell-free polypeptide synthesis at high temperature (Uzawa et al. 1993; Terui et al. 2005); for an in vitro process observed in cell extracts and for further details of the roles of polyamines in thermophiles, see Chap. 12.

The quantity of long-chain and branched polyamines in the cells of hyperthermophiles increases at high growth temperatures. For instance, cells of *Thermococcus kodakarensis* contain high levels of spermidine at 60 °C. The level of branched polyamines increases at high temperatures (Morimoto et al. 2010; Okada et al. 2014). Moreover, thermophiles that have lost the ability to produce long-chain and branched polyamines [as the result of gene disruption(s)] cannot grow at high temperatures (Ohnuma et al. 2005; Morimoto et al. 2010). Collectively, these results indicate a key role for long-chain and branched polyamines in cellular tolerance to high temperature.

## 2.3 Polyamine Biosynthesis in Thermophiles

Polyamines are synthesized from amino acids such as arginine, ornithine, lysine, and methionine (Rhee et al. 2007). In most eukaryotes, putrescine and cadaverine are synthesized directly from ornithine and lysine by ornithine decarboxylase and lysine decarboxylase, respectively (Wallace et al. 2003; Schneider and Wendisch 2011). Putrescine biosynthesis pathways in plants and some bacteria synthesize putrescine from arginine via agmatine (Tabor and Tabor 1985; Imai et al. 2004; Yang and Lu 2007). In this pathway, agmatine is synthesized by arginine decarboxylase, and agmatine is converted to putrescine either by agmatine ureohydrolase (agmatinase) or a combination of agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase (pathway I; Fig. 2.2e). Longer polyamines, such as spermidine and spermine, are produced by addition of an aminopropyl group from decarboxylated *S*-adenosyl methionine (dcSAM). However the thermophilic bacterium *Thermus thermophilus* and the hyperthermophilic archaeon *T. kodakarensis* possess a distinct polyamine biosynthetic pathway (pathway II; Fig. 2.2e) (Ohnuma et al. 2005; Morimoto et al. 2010), and synthesize spermidine from agmatine via aminopropylagmatine (a process catalyzed by aminopropyl transferase and ureohydrolase). The long-chain and branched polyamines are synthesized by the addition of an aminopropyl group donated by dcSAM, and the enzymatic addition of the aminopropyl group has been identified recently (Okada et al. 2014). Further details of the key enzymes involved in the synthesis of long-chain and branched polyamides in thermophiles are given next.

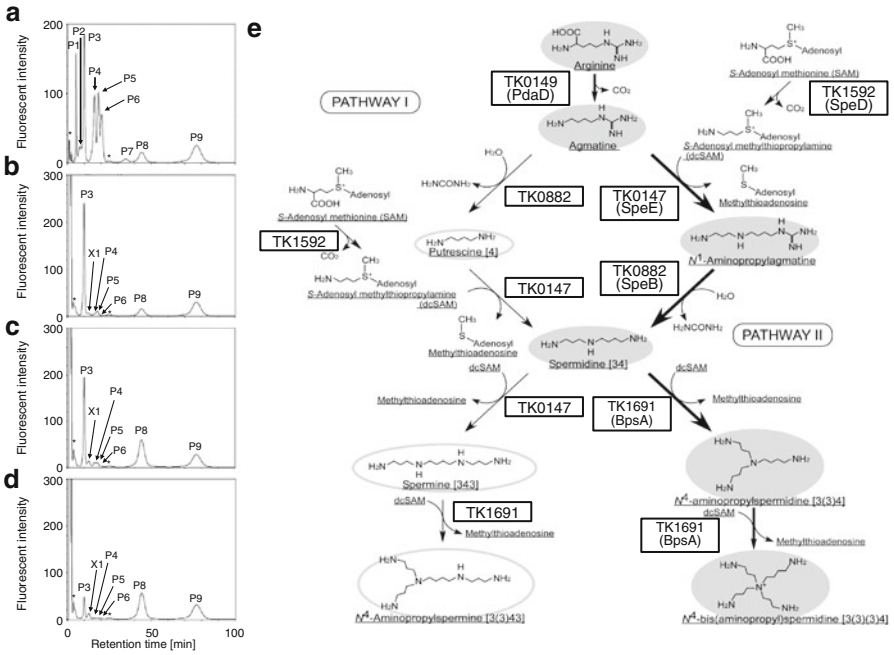


**Fig. 2.2** Intracellular polyamines (**a**–**d**) and biosynthetic pathway of polyamines (**e**) in *T. kodakarensis*. Intracellular polyamines were extracted from *T. kodakarensis* KU216 cells grown at 60, 85, and 93 °C and were analyzed by HPLC. The eluted polyamines were labeled with *o*-phthaldehyde and detected with a fluorescence detector. **a** Peak standards of chemically synthesized polyamines; **b** polyamines extracted from cells grown at 60 °C; **c** sample from cells grown at 85 °C; **d** sample from cells grown at 93 °C. *P1* putrescine [4], *P2 S*-adenosyl methylthiopropylamine (decarboxylated *S*-adenosyl methionine), *P3* spermidine [34], *P4* agmatine, *P5* tris(3-aminopropyl)amine [3(3)3], *P6* spermine [343], *P7* $N^1$-aminopropylagmatine (ApAGM), *P8* $N^4$-bis(aminopropyl)spermidine [3(3)(3)4], *P9* caldohexamine [33333] used as internal standard, *X1* unknown peak. *Asterisks* indicate noise created by the buffer. **e** Compounds highlighted by *shading* were detected in the *TK0147, TK0882,* or *TK1691* gene disruptant of *T. kodakarensis*. *Bold arrows* represent the main pathway for the production of spermidine and $N^4$-bis(aminopropyl) spermidine in *T. kodakarensis. Pathway I* is spermidine biosynthetic pathways found in bacteria and plants; *pathway II* is uniquely found in thermophilic bacteria and euryarchaeotal hyperthermophiles. Putrescine, spermine, and $N^4$-aminopropylspermine are produced only in vitro by agmatine ureohydrolase (TK0882), spermidine synthase (TK0147), and branched-chain polyamine synthase (TK1691), respectively

### *2.3.1* S-*Adenosylmethionine Decarboxylase*

The elongation of polyamine is catalyzed by aminopropyl transferase in the presence of the donor of the aminopropyl group (dcSAM) described above. *S*-Adenosylmethionine (SAM) decarboxylase is designated as SpeD. Most SAM

decarboxylases that produce dcSAM belong to the pyruvoyl-dependent group of enzymes. These enzymes are expressed as inactive proenzymes that self-cleave at a serine residue to produce two active subunits (subunits α and β). The $NH_2$ terminus of the serine residue is then converted to a pyruvoyl group by deammoniation (van Poelje and Snell 1990). SAM decarboxylases have been classified into two groups, which are in turn each divided into two subgroups according to Bale and Ealick (2010). Bacterial and archaeal SAM decarboxylases belong to group 1 and the eukaryotic enzymes belong to group 2. The SAM decarboxylases belonging to group 1a (such as those from the gram-negative bacterium *Escherichia coli*) require a metal ion for activity whereas those belonging to group 1b (e.g., those from the gram-positive bacterium *Bacillus subtilis*) do not require a metal ion. Genes that code for SAM decarboxylases are found within the genomes of many hyperthermophilic microbes (Kim et al. 2000; Giles and Graham 2008). Several studies have helped elucidate the crystal structures, catalytic activities, and other properties of SAM decarboxylases from hyperthermophiles, although little is still known about the expression of the genes that encode these enzymes or what regulates their catalytic activity. Group 1b enzymes are found in the hyperthermophilic bacteria *Thermotoga maritima* (TM0655) and *Aquifex aeolicus* (aq254), and in the hyperthermophilic methanogenic archaeon *Methanocaldococcus jannaschii* (MJ0315) (Lu and Markham 2004; Bale and Ealick 2010); the active form of these enzymes is an $(\alpha\beta)_2$ dimer. SAM decarboxylase from the hyperthemoplic acidophilic archaeon *Sulfolobus solfataricus* is a monomeric protein (Cacciapuoti et al. 1991). Although SAM decarboxylases from mammalian and yeast cells are specifically (and strongly) activated by putrescine, those from *S. solfataricus* and *M. jannaschii* are not altered by the addition of putrescine or a divalent cation (Cacciapuoti et al. 1991; Kim et al. 2000). The TK1592 gene found in the hyperthermophilic archaeon *T. kodakarensis* is an orthologue of MJ0315 and heterologously expressed TK1592 protein in *E. coli* forms the $(\alpha\beta)_2$ complex (unpublished data). TK1592 is expressed over a wide range of growth temperatures (i.e., from 60 to 93 °C) and is strongly induced at 93 °C. This increase in expression at high temperatures is likely to increase enhance polyamine synthesis via an increase in the production of the propylamine donor.

Archaeal arginine decarboxylases are also pyruvoyl-dependent enzymes. Little structural similarity between arginine decarboxylases and SAM decarboxylases is found in members of the Euryarchaeota (Fig. 2.3), although there are two paralogues of the SAM decarboxylase in *S. solfataricus*, which belong to the Crenarchaeota (Giles and Graham 2008) and are coded for by the SSO0585 gene (SAM decarboxylase) and SSO0536 gene (arginine decarboxylase). The SSO0536 gene is not associated with SAM decarboxylase activity, and the SSO0585 gene does not give rise to arginine decarboxylase activity. However, a chimeric protein composed of the α-subunit of SSO0536 and the β-subunit of SSO0585 had arginine decarboxylase activity (Giles and Graham 2008), indicating that residues responsible for substrate specificity are present in the N-terminal domain. Based on these results, it is likely that arginine decarboxylase evolved from an SAM decarboxylase enzyme in members of the Crenarchaeota.

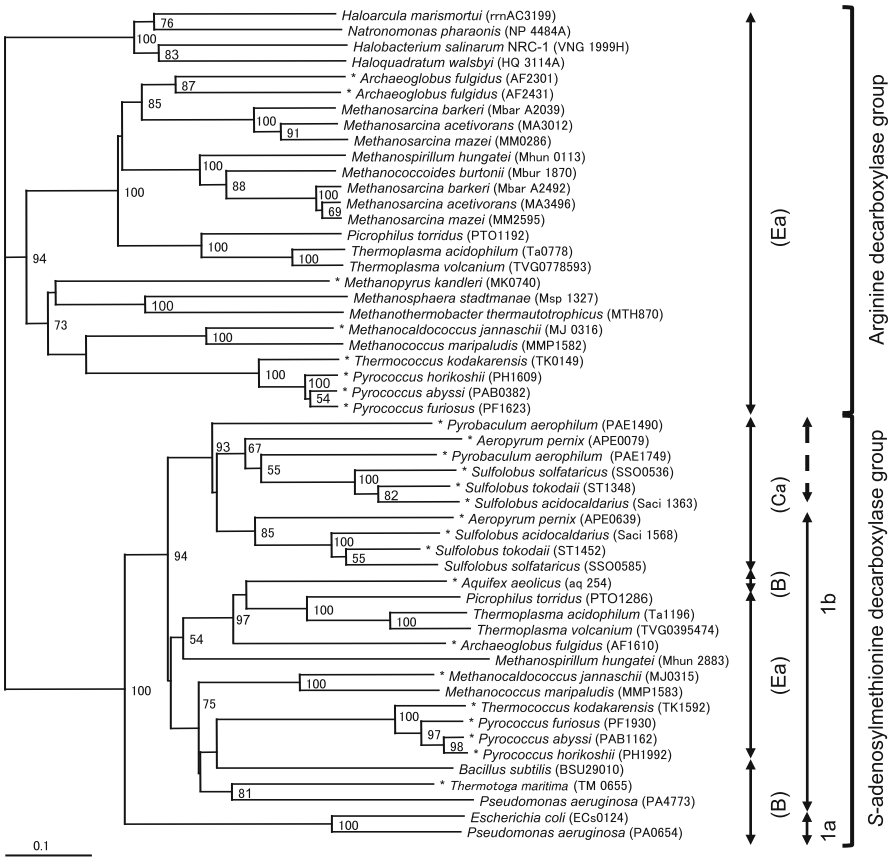


**Fig. 2.3** Phylogenetic tree based on amino acid sequences of *S*-adenosyl methionine decarboxylases and arginine decarboxylases. *Codes* in *parentheses* are open reading frame (ORF) numbers. This tree was constructed using the neighbor-joining method provided by DNA Data Bank of Japan (DDBJ). Bootstrap re-sampling was performed 1,000 times, and only values observed in more than 50 % of the replicas are shown at branching points. *Scale bar* indicates one substitution per 100 amino acids; *asterisks* indicate hyperthermophiles. *1a* and *1b* indicate groups classified by the previous study (Bale and Ealick 2010). *B* Bacteria, *Ea* Euryarcaeota, *Ca* Crenarchaeota

## 2.3.2 *Arginine Decarboxylase*

Arginine decarboxylases, which produce agmatine from arginine, have been divided into three different types: pyridoxal 5′-phosphate (PLP)-dependent arginine decarboxylases and pyruvoyl-dependent arginine decarboxylases. Pyruvoyl-dependent arginine decarboxylases (PdaD) have been found in the hyperthermophilic Archaea *M. jannaschii* (MJ0316) and *T. kodakarensis* (TK0149) (Graham et al. 2002; Tolbert et al. 2003; Fukuda et al. 2008). TK0149 forms an $(\alpha\beta)_6$ complex whereas MJ0316 forms an $(\alpha\beta)_3$ complex (Graham et al. 2002; Fukuda et al. 2008), and both enzymes

catalyze the decarboxylation of arginine, but not that of ornithine or lysine. A *T. kodakarensis* mutant that lacked TK0149 was only able to grow in the presence of agmatine (Fukuda et al. 2008), an essential metabolite in this species. Archaeal cells use agmatine to synthesize agmatidine, an agmatine-conjugated cytidine found at the anticodon wobble position of archaeal $tRNA^{Ile}$ (Ikeuchi et al. 2010). Agmatine is an essential precursor of both agmatidine and polyamines.

### 2.3.3 Agmatine Ureohydrolase and Aminopropylagmatine Ureohydrolase

A biosynthetic pathway that produces spermidine via aminopropylagmatine has been identified in the thermophilic bacterium *Thermus thermophilus* (Ohnuma et al. 2005). In *T. thermophilus,* $N^1$-aminopropylagmatine is synthesized by aminopropyl transferase encoded by *speE* (*TT0339*), and then spermidine is synthesized by $N^1$-aminopropylagmatine ureohydrolase encoded by *speB* (*TT0338*). In hyperthermophilic Archaea, the same biosynthetic pathway is proposed, as shown in Fig. 2.2e. According to genome analysis, the archaeon *T. kodakarensis* possesses three homologues of agmatine ureohydrolase (TK0240, TK0474 and TK0882) (Fukui et al. 2005). Of these, only TK0882 gene disruptants were unable to grow at 85 and 93 °C, but their ability to grow was partially restored when spermidine was exogenously supplied. Intriguingly, agmatine and $N^1$-aminopropylagmatine accumulated in the cytoplasm of the TK0882 gene disruptant. Purified TK0882 has a 54-fold-lower $K_m$ value for $N^1$-aminopropylagmatine than for agmatine, indicating that it is a $N^1$-aminopropylagmatine ureohydrolase (SpeB). These results imply that spermidine is usually synthesized from agmatine via $N^1$-aminopropylagmatine. The roles of TK0240 and TK0474, which may be involved in metabolic activities other than polyamine synthesis, remain unclear.

### 2.3.4 Aminopropyl Transferase

Aminopropyl transferases are involved in the elongation of polyamines. The hyperthermophilic archaeon *Pyrococcus furiosus* (which is closely related to *Pyrococcus horikoshii* and *T. kodakarensis*) possesses an enzyme known as PF0127, which has aminopropyl transferase activity (Cacciapuoti et al. 2007). PF0127 has a broad substrate specificity and its amine acceptors are agmatine, 1,3-diaminopropane, putrescine, cadaverine, and *sym*-nor-spermidine. Although optimal catalytic activity was observed with cadaverine (~20-fold higher than that for agmatine), agmatine was the preferred substrate on the basis of the $k_{cat}/K_m$ value (~20-fold higher than that for cadaverine). Spermidine, cadaverine, and *sym*-nor-spermidine are the primary polyamines in *P. furiosus*, and PF0127 seems to act as an agmatine aminopropyl transferase in vivo. Aminopropylcadaverine is speculated to be involved in stress tolerance under unfavorable environmental conditions (Cacciapuoti et al. 2007).

*T. kodakarensis* TK0147 has been annotated as a spermidine synthase and shares sequence identity with PF0127 (Morimoto et al. 2010). The TK0147 gene disruptant accumulated large amounts of agmatine and smaller amounts of putrescine and showed a slow growth rate at elevated temperatures (of 85 and 93 °C). Purified recombinant TK0147 possesses a $K_m$ value for agmatine that is 427-fold lower than that for putrescine, suggesting that TK0147 mainly functions as an aminopropyl transferase in the production of $N^1$-aminopropylagmatine. In *T. kodakarensis*, spermidine is mainly produced from agmatine via $N^1$-aminopropylagmatine by TK0147 and TK0882. The majority of spermidine in this species produced in this way. $N^4$-Bis(aminopropyl)spermidine was detected in the TK0147 gene disruptant, indicating that TK0147 does not function as an aminopropyl transferase in the production of longer branched chain polyamines (Morimoto et al. 2010). Another aminopropyl transferase has recently been identified in *T. kodakarensis*, as described next.

### 2.3.5 Branched-Polyamine Synthases

An enzyme exhibiting branched-polyamine synthase activity has recently been identified and named TK1691 (Okada et al. 2014). Recombinantly expressed TK1691 catalyzes the synthesis of $N^4$-bis(aminopropyl)spermidine from spermidine and dcSAM via $N^4$-aminopropylspermidine [3(3)4]. We tentatively designate TK1691 as branched-polyamine synthase A (BpsA). TK1691 also produces $N^4$-aminopropylspermine [3(3)43] from spermine [343]. Sequence comparisons using database sequences compiled from various microorganisms indicate that TK1691 and its orthologues are distinct from other known aminopropyl transferases that produce thermospermine, spermidine, and spermine. TK1691 and its orthologues are characterized by a conserved region that recognizes dcSAM aminopropyl but lack the general polyamine-binding motif found in spermidine synthases and spermine synthases. Furthermore, TK1691 orthologues have been identified in both archaeal and bacterial hyperthermophiles (Fig. 2.4). By contrast, aminopropyl transferases that produce spermidine, thermospermine, and spermine, spermidine/spermine synthase homologues including *E. coli* SpeE and *T. kodakarensis* TK0147, have been identified in various organisms. In the phylogenetic tree, we classified aminopropyl transferases into four groups. One of these groups, tentatively designated as group A, contains the thermophilic TK1691 orthologues (*P. furiosus* PF1111, *Methanotorris igneus* Metig0730, *T. thermophilus* TTHA0539). The other group, group B, contains TK0147 orthologues (*E. coli* SpeE, *Aquifex aeolicus* aq062, *P. furiosus* PF0127). Several other aminopropyl transferases have been annotated as thermospermine synthases and designated as aminotransferase group C: Igni0633 from *Ignicoccus hospitalis*, Hbut0057 and Hbut0383 from *Hyperthermus butylicus*, and At5g19530 from *Arabidopsis thaliana* (Knott et al. 2007; Knott 2009). Phylogenetic analyses suggest that there are several types of aminopropyl transferases. As indicated in the tree for *M. jannaschii*, one of the archaeal hyperthermophiles has two TK1691 orthologues, MJ1273 and MJ0675. MJ1273 shows

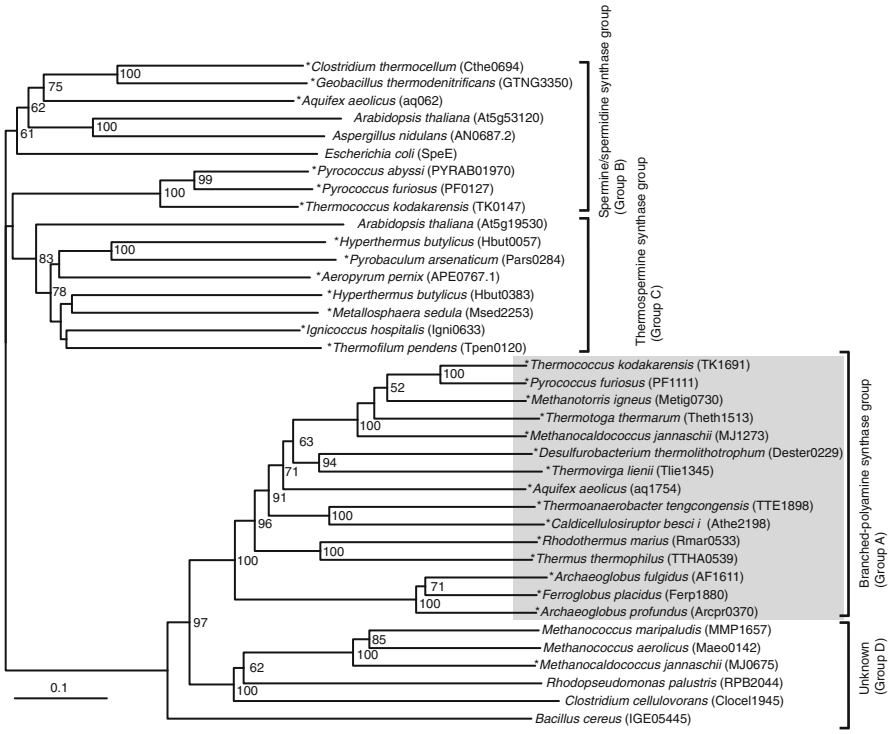


**Fig. 2.4** Phylogenetic tree based on amino acid sequences of aminotransferases was constructed according to the method used to construct the tree in Fig. 2.3

high homology to TK1691 and belongs to aminopropyl transferase group A. MJ1273 is assumed to be the enzyme responsible for the synthesis of branched-chain polyamines. In contrast, MJ0675 is located on the different branch of the tree and has been tentatively designated as belonging to group D. MMP1657 and Maeo0142 from *Methanococcus maripaludis* and *Methanococcus aeolicus,* respectively, have homology to MJ0675 and are predicted to be RNA methylases. Methyltransferases transfer a methyl group from SAM to an acceptor, and these enzymes and aminopropyl transferases are thought to be derived from a common ancestral protein. Based on these results, TK1691 appears to be a hitherto uncharacterized aminopropyl transferase involved in the synthesis of branched-chain polyamines.

## 2.4 Conclusion

Polyamines are important, physiologically active substances in microbial cells. For thermophilic species, the intracellular concentrations of long-chain and branched polyamines are higher than in other organisms and are important for growth at high

temperatures. Although the biosynthesis of branched polyamines is stimulated by high temperature, the molecular mechanisms by which this occurs have yet to be characterized. Further studies are needed to elucidate the roles of long-chain and branched polyamines in hyperthermophiles (see also Chap. 12).

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# Chapter 3
# Regulation and Diversity of Polyamine Biosynthesis in Plants

**Taku Takahashi and Wurina Tong**

**Abstract** In higher plants, the main route for putrescine synthesis may be from arginine. Higher accumulation of putrescine under biotic and abiotic stress conditions is attributed at least in part to stress-inducible expression of arginine decarboxylase genes. Regulation of spermidine synthesis involves translational control of the *S*-adenosylmethionine decarboxylase mRNA, which is responsive to cellular polyamine levels. Genes encoding *S*-adenosylmethionine decarboxylase and spermine synthase are also responsive to environmental stimuli or show preferential expression in certain organs. Stress-inducible expression of these genes involves plant hormone signaling of abscisic acid (ABA) or methyl jasmonate (MeJA). Thermospermine synthase is widely distributed in the plant kingdom but has a unique role in the repression control of xylem differentiation in higher plants. Expression of the gene for thermospermine synthase is under negative feedback control by thermospermine. Putrescine, spermidine, and cadaverine are also essential precursors for alkaloid biosynthesis in some plant species. Polyamines occur in various aspects of plant growth as a conjugated form with cinnamic acids and proteins. Genes responsible for these conjugations have been increasingly identified.

**Keywords** ABA • Arabidopsis • Auxin • MeJA • Plant hormone • Transcription factor • Translation • Xylem

## 3.1 Introduction

A number of early studies on plant polyamines have documented that increased biosynthesis of polyamines is associated with the growth, regeneration, and responses to external environmental stimuli of plants (Flores and Galston 1982; Evans and Malmberg 1989; Galston and Sawhney 1990; Kumar et al. 1997). Detailed information on plant polyamine physiology and biochemistry has previously been

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compiled (Slocum and Flores 1991). More recently, molecular genetic studies using transgenic plants with altered polyamine biosynthesis and those using mutants that are defective in polyamine biosynthesis, especially in a model plant system, *Arabidopsis thaliana*, have provided convincing evidence that polyamines act as critical modulators of various physiological processes during plant growth and development (Kusano et al. 2008; Alcázar et al. 2010; Takahashi and Kakehi 2010). In *Arabidopsis*, whose whole genome sequence has been determined and all polyamine biosynthetic genes have been identified (Table 3.1), some of these genes are responsive to specific stress conditions and plant hormones or show tissue-specific expression. However, because of their versatile nature as small polycations, ability to neutralize negative charges, and ubiquitous distribution in plant cells including the cytoplasm, vacuoles, chloroplasts, and mitochondria, understanding of the function of polyamines in plant cells is still far from complete.

The cellular content of the three major polyamines putrescine, spermidine, and spermine differs among species, with putrescine and spermidine being particularly abundant and spermine less abundant in plant cells. It has also been suggested that putrescine is a negative regulator whereas spermidine and spermine are positive regulators of cellular amino acid metabolism (Mattoo et al. 2010). Additionally, although being much less abundant, thermospermine, a structural isomer of spermine, is widely detected in the plant kingdom and likely functions as a plant hormone in higher plants (Takano et al. 2012). In this chapter, we focus on the regulation of polyamine biosynthesis with reference to their cellular function in higher plants.

**Table 3.1** List of polyamine biosynthetic genes in *Arabidopsis*

| Catalytic function | Gene name | Gene ID | Factors that enhance expression |
|---|---|---|---|
| Arginine decarboxylase | *ADC1* | At2g16500 | Cold |
| | *ADC2* | At4g34710 | Drought, osmosis, salt, wounding, cold, ABA, MeJA<br>$K^+$ deficiency, bacterial pathogen |
| Agmatine iminohydrolase | *AIH* | At5g08170 | |
| *N*-Carbamoylputrescine amidohydrolase | *CPA* | At2g27450 | |
| Arginase | *ARGAH1* | At4g08900 | |
| | *ARGAH2* | At4g08870 | MeJA, pathogen |
| *S*-Adenosylmethionine decarboxylase | *SAMDC1* | At3g02470 | |
| | *SAMDC2* | At5g15950 | Cold, heat shock |
| | *SAMDC3* | At3g25570 | |
| | *SAMDC4/BUD2* | At5g18930 | Thermospermine depletion, auxin |
| Spermidine synthase | *SPDS1* | At1g23820 | Drought, ABA |
| | *SPDS2* | At1g70310 | Cytokinin |
| Spermine synthase | *SPMS* | At5g53120 | Drought, ABA, heat shock |
| Thermospermine synthase | *ACL5* | At5g19530 | Thermospermine depletion, auxin |
| Deoxyhypusine synthase | *DHS* | At5g05920 | |

## 3.2 Putrescine Biosynthesis

Putrescine is important not only as an essential substrate for the synthesis of higher polyamines but also as a stress signal molecule. Putrescine is synthesized from arginine or ornithine (Fig. 3.1). Although the conversion of ornithine to putrescine by ornithine decarboxylase (ODC), namely the ODC pathway, is a rate-limiting step for polyamine synthesis in animals and fungi, the arginine decarboxylase (ADC) pathway may be a primary route for putrescine synthesis in plants. In the ADC pathway, arginine is converted to agmatine by ADC and agmatine is then converted to putrescine via an *N*-carbamoylputrescine intermediate by two-step reactions involving agmatine deiminase/iminohydrolase (AIH) and *N*-carbamoylputrescine amidase/amidohydrolase (CPA). The gene that was initially identified as a nitrilase-like protein and named *NLP1* in *Arabidopsis* has been shown to encode CPA, and *NLP1/CPA* expression is detected in all organs investigated (Piotrowski et al. 2003). The *AIH* gene has also been identified in *Arabidopsis* (Illingworth et al. 2003; Janowitz et al. 2003). Comparison of genome sequences suggests that the ADC pathway in plants was inherited from the cyanobacterial ancestor of the chloroplast (Illingworth et al. 2003), but single-celled green algae have lost the ADC pathway and are dependent on the ODC pathway as are other eukaryotes, although the moss *Physcomitrella patens* and some higher plant species have lost ODC (Fuell et al. 2010). The *Arabidopsis* genome contains no gene for ODC (Hanfrey et al. 2001) but has

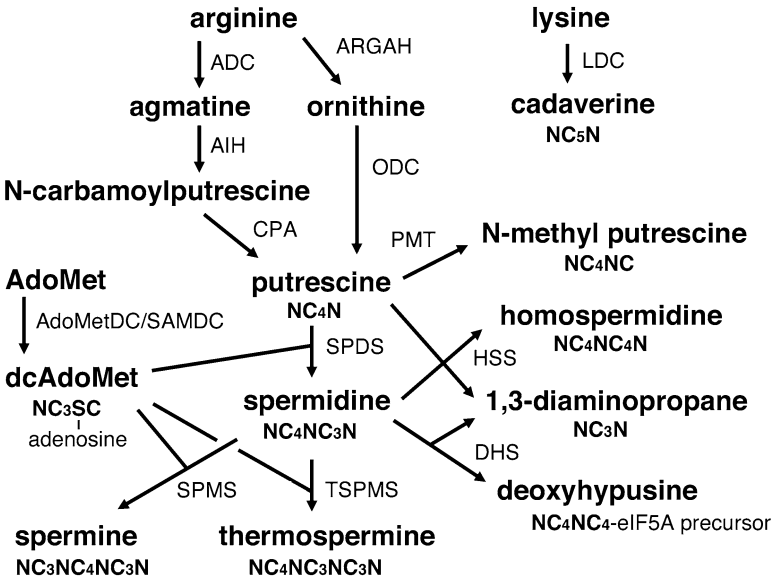


**Fig. 3.1** Polyamine biosynthetic pathways in plants. Aminopropyl and aminobutyl groups in polyamines are shown as $NC_4$ and $NC_3$, respectively

two genes for ADC, *ADC1* and *ADC2*. Although both two genes are responsive to cold treatment (Hummel et al. 2004; Cuevas et al. 2008), *ADC2* expression is highly induced by various abiotic stresses such as dehydration, mechanical wounding, high salinity, and $K^+$ deficiency (Soyka and Heyer 1999; Pérez-Amador et al. 2002; Urano et al. 2003, 2004; Armengaud et al. 2004). Most of these responses are mediated by abscisic acid (ABA), a major plant hormone mediating the adaptation to stress, and wounding involves methyl jasmonate (MeJA) signaling. The *ADC2* promoter contains several ABA-responsive elements (ABREs), and its response to dehydration is ABA dependent (Alcázar et al. 2006a, b). *ADC2* expression is also increased by bacterial pathogen infection (Kim et al. 2013), indicating a protective role of putrescine against both biotic and abiotic stresses. The double-knockout mutant of *ADC1* and *ADC2* has been shown to be lethal at the embryo stage (Urano et al. 2005), indicating that putrescine is essential for plant survival. In cacao, both *ADC* and *ODC* genes are responsive to drought, wounding, and pathogen infection (Bae et al. 2008). These genes in tobacco are upregulated by MeJA treatment (Biondi et al. 2001), but in rice *OsADC1* expression and the free putrescine content are significantly reduced by MeJA (Peremarti et al. 2010), suggesting different responses between plant species.

Arginine is also hydrolyzed to produce ornithine and urea by arginase, which is also known as arginine amidohydrolase (ARGAH; Fig. 3.1). Although the ARGAHs-knockout mutant of *Arabidopsis* accumulates significantly increased levels of polyamines and nitric oxide (NO) and further shows enhanced tolerance to multiple abiotic stresses such as drought, high salt, and freezing, ARGAH-overexpressing lines exhibit reduced tolerance to these stresses and reduced levels of polyamines and NO (Shi et al. 2013), suggesting the importance of arginine metabolism in the control of polyamine and NO levels and the stress response in *Arabidopsis*. *ARGAH2* expression is induced following MeJA treatment (Brownfield et al. 2008) and inoculation with the necrotrophic pathogen (Brauc et al. 2012).

It is known that enzyme activities of ODC and ADC are inhibited by difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA), respectively. Many experiments have examined the physiological effect of these inhibitors on the plant growth and the biosynthesis of polyamines. However, some of the data are still difficult to interpret. The outcome of in vivo experiments using these inhibitors should be carefully evaluated because they may have unexpected side effects (Smith 1990).

## 3.3 Spermidine Biosynthesis

Spermidine is synthesized from putrescine by the transfer of an aminopropyl group from decarboxylated *S*-adenosylmethionine (dcSAM), a reaction catalyzed by spermidine synthase (SPDS; Fig. 3.1). In the *Arabidopsis* genome, because of the presence of numerous large duplicated segments in chromosomes (Vision et al. 2000), there are also two genes encoding SPDS, *SPDS1* and *SPDS2*. A single-knockout mutant of either *SPDS1* or *SPDS2* shows no obvious phenotype, but the double mutant


**Fig. 3.2** Mature embryos in the wild-type (Col-0) and *spds1 spds2* double-mutant seed of *Arabidopsis*

of these two genes is lethal during seed development, indicating that spermidine is essential for survival of embryos (Imai et al. 2004b) (Fig. 3.2). Expression of these genes is detected in all organs examined and *SPDS2* is moderately upregulated by cytokinin (Hanzawa et al. 2002), whereas *SPDS1* is highly induced by drought stress in an ABA-dependent manner (Alcázar et al. 2006a). Neither of these genes is downregulated by external spermidine (Kakehi et al. 2010).

SPDS activity is inhibited by a competitive inhibitor cyclohexylamine (CHA). In early studies on plant polyamines, treatments of thin-layered tissue cultures of tobacco with CHA have been shown to inhibit floral initiation but to be reversed by spermidine application (Kaur-Sawhney et al. 1988). On the other hand, CHA treatment results in a drastic increase in the level of purescine but has little or no effect on the level of spermidine and spermine in other plant systems such as the vegetative organs of *Arabidopsis* (Stes et al. 2011) and germinating pollen of kiwifruit (Scoccianti et al. 2013).

Because the biosynthesis of spermidine and higher polyamines by aminopropyl transferases utilizes dcSAM as an aminopropyl donor, the production of dcSAM by the action of *S*-adenosylmethionine decarboxylase (AdoMetDC/SAMDC) is a key step in polyamine production (Fig. 3.1). Overexpression of the *Datura AdoMetDC/SAMDC* cDNA in transgenic rice has been shown to cause an increase in the level of spermidine in leaves and also that of spermine in seeds (Thu-Hang et al. 2002). There are many reports indicating that the level of polyamines, especially that of spermidine, is increased during pollen germination and tube growth. In accordance with this phenomenon, increased expression of *AdoMetDC/SAMDC* genes has been observed in germinating pollens of some plants, such as tomato (Song et al. 2001) and kiwifruit (Antognoni and Bagni 2008). Expression of an *AdoMetDC/SAMDC* gene is upregulated by MeJA treatment of tobacco thin-layer explants (Biondi et al. 2001).

In mammals, spermidine and spermine negatively regulate the translation of AdoMetDC/SAMDC with a small upstream open reading frame (uORF) of its mRNA that encodes a conserved hexapeptide, 'MAGDIS' (Hill and Morris 1993; Ruan et al. 1996). A similar homeostatic circuit has been found in the AdoMetDC/SAMDC translation in *Arabidopsis* (Hanfrey et al. 2005; see Chap. 9). The *Arabidopsis* genome contains four genes encoding AdoMetDC/SAMDC, among which *AdoMetDC/SAMDC1* and *AdoMetDC/SAMDC2* contain a highly conserved pair of overlapping uORFs (Franceschetti et al. 2001). These overlapping uORFs are also present in the 5′-leader sequence of *AdoMetDC/SAMDC* in monocots and gymnosperms, suggesting a conserved regulatory mechanism of the *AdoMetDC/SAMDC* translation (Franceschetti et al. 2001).

AdoMetDC/SAMDC activity is competitively inhibited by methylglyoxal bis(guanylhydrazone) (MGBG). However, although there is much evidence showing that MGBG negatively affect plant growth and development, its specificity is also questioned. A study suggests that MGBG can have a negative effect on plant growth by enhancing, rather than by reducing, the cellular polyamine pool (Scaramagli et al. 1999).

The aminopropyl transfer from dcSAM results in the release of 5′-methylthioadenosine (MTA), which is rapidly metabolized and recycled to the SAM precursor methionine in a cyclic pathway known as the methionine salvage cycle (Sauter et al. 2013). MTA is also released from SAM in the biosynthesis of ethylene and nicotianamine and considered a toxic metabolite because of product inhibition. A study has shown that MTA affects the synthesis of polyamines (Waduwara-Jayabahu et al. 2012).

Spermidine is an essential precursor for deoxyhypusine synthesis, and the absolute requirement for spermidine may be attributed at least to its role as an aminobutyl donor for the hypusine modification of the eukaryotic translation initiation factor 5A (eIF5A; see Chap. 10). Deoxyhypusine synthase (DHS) catalyzes transfer of the aminobutyl moiety of spermidine to the ε-amino group of highly conserved lysine-50 in the eIF5A precursor protein (Fig. 3.1). The deoxyhypusine-eIF5A is further converted to the active hypusine-eIF5A, which is essential for the transport of newly transcribed mRNAs from nucleus to cytoplasm (Chattopadhyay et al. 2003). A mutant of the *DHS* gene in *Arabidopsis* is female gametophyte lethal and cannot be homozygous diploid (Pagnussat et al. 2005).

The aminobutyl moiety of spermidine is also transferred to putrescine by the action of homospermidine synthase (HSS; Fig. 3.1). The resulting homospermidine is an essential precursor in the biosynthesis of pyrrolizidine alkaloids that serve as defense compounds against insect predators and occur in a number of families including Asteraceae, Boraginaceae, and Orchidaceae (Ober et al. 2003). A high degree of sequence identity between *DHS* and *HSS* genes suggests that *HSS* evolved by gene duplication from *DHS* (Ober and Hartmann 1999), which may have occurred at least four times in separate angiosperm lineages (Reimann et al. 2004).

In parallel with conversion to spermidine, putrescine also serves as a substrate for the biosynthesis of tropane alkaloids such as nicotine, scopolamine, and cocaine in the nightshade family (Solanaceae). They function in deterring herbivores (Nathanson et al. 1993). Putrescine *N*-methyltransferase (PMT) is the first specific

enzyme of the synthesis of these alkaloids (Fig. 3.1), and the overall similarity between PMT and SPDS suggests the evolutionary origin of PMT from ubiquitous SPDS (Hashimoto et al. 1998, see Chap. 17). Furthermore, PMT activity has shown to be generated by genetically exchanging few amino acids of the recombinant SPDS protein from *Datura* and *Arabidopsis* (Junker et al. 2013).

## 3.4 Spermine Biosynthesis

Spermidine is further converted to spermine by the action of spermine synthase (SPMS; Fig. 3.1). The arrangement of exons and introns and the size of each exon are almost completely conserved between *SPDS* and *SPMS* gene families in plants but are far different from those of animals and fungi, suggesting that *SPMS* has evolved from *SPDS* independent from that of animals and fungi (Minguet et al. 2008; Shao et al. 2012). No genes for SPMS are found in ferns, mosses, and algae (Minguet et al. 2008; Pegg and Michael 2010). Monocot *SPMS* genes contain an additional exon that encodes a PEST-rich region at the C-terminus, which might be involved in the control of SPMS protein degradation in monocots (Rodríguez-Kessler et al. 2010). Because the knockout mutant of *SPMS* in *Arabidopsis* displays wild-type morphology under normal growth condition, spermine is not essentially required for survival (Imai et al. 2004a). However, the mutant is more sensitive to drought and salt stresses than the wild type (Yamaguchi et al. 2007). In accordance with this mutant phenotype, *SPMS* expression in *Arabidopsis* seedlings is increased by treatments with ABA (Hanzawa et al. 2002). Similarly, expression of the maize *SPMS* gene, formerly named *ZmSPDS2* (Rodríguez-Kessler and Jiménez-Bremont 2008), is upregulated by ABA (Jiménez-Bremont et al. 2007). The increase in the level of spermine by ABA has also been found in other plants, such as grass pea (Xiong et al. 2006) and wheat (Kovács et al. 2010). In addition, a study on the heat-shock response in *Arabidopsis* shows that *SPMS* and *SAMDC2* are induced at the earliest stage of heat-shock treatment, followed by the induction of *ADC2* and the increase in spermine content (Sagor et al. 2013).

A previous work in *Arabidopsis* has shown that both SPDS1 and SPDS2 physically interact with SPMS and form a polyamine metabolon, a multiprotein complex assembly, which is probably responsible for efficient production of spermine (Panicot et al. 2002). A more recent study has suggested that these heterodimer complexes take place preferentially inside the nucleus (Belda-Palazón et al. 2012).

## 3.5 Thermospermine Biosynthesis

Thermospermine was first discovered in thermophilic bacteria (Oshima 2007) (see Chap. 2). In plants, aminopropyl transferase activities responsible for biosynthesis of thermospermine as well as longer polyamines have been detected in alfalfa

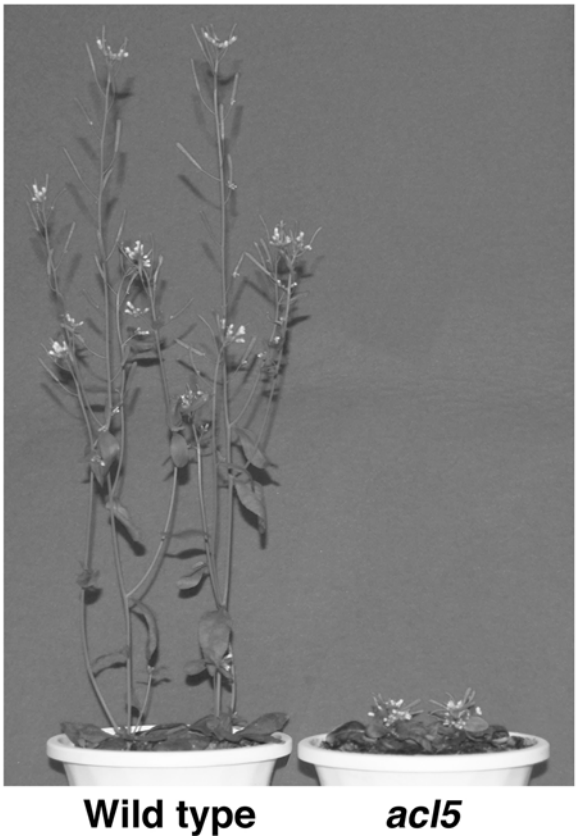


**Fig. 3.3** Thirty-five-day-old plants of the wild type (L*er*) and *acl5* mutant of *Arabidopsis*

(Bagga et al. 1997). On the other hand, an *Arabidopsis* mutant that displays excess differentiation of xylem vessels and severe dwarfism was isolated and named *acaulis5* (*acl5*) after its no-stem phenotype (Hanzawa et al. 1997, Fig. 3.3). Although the gene responsible for the *acl5* phenotype was initially misidentified as encoding SPMS (Hanzawa et al. 2000), it was later proved to encode thermospermine synthase (TSPMS) (Knott et al. 2007; Kakehi et al. 2008). *ACL5/TSPMS*-like genes are widely distributed in the plant kingdom, including diatoms, water molds, brown algae, green algae, mosses, liverworts, ferns, and gymnosperms, but are not found in animals and fungi (Knott et al. 2007; Pegg and Michael 2010; Takano et al. 2012). A phylogenetic study suggests that TSPMS was acquired by an ancestor of the plant lineage through horizontal gene transfer from Archaea or Bcteria (Minguet et al. 2008). Alternatively, it is also suggested that TSPMS was transferred from the cyanobacterial ancestor of the chloroplasts to the host nucleus (Fuell et al. 2010).

The content of thermospermine in individual plants is several fold lower than that of spermine in *Arabidopsis* (Naka et al. 2010; Rambla et al. 2010), mainly because *ACL5/TSPMS* expression is sharply limited to xylem precursor cells during vascular formation (Clay and Nelson 2005; Muñiz et al. 2008). Because the mutated

*acl5/tspms* transcript level in the *acl5* mutant is much higher than the *ACL5/TSPMS* transcript level in the wild type (Hanzawa et al. 2000) and *ACL5/TSPMS* expression is drastically reduced by thermospermine treatment (Kakehi et al. 2008), *ACL5/TSPMS* appears to be under negative feedback control by thermospermine. Although its molecular mechanism remains unknown, a basic helix-loop-helix (bHLH) transcription factor SAC51 has been suggested to be involved in the repression of *ACL5/TSPMS* expression (Imai et al. 2006). *SAC51* was identified as a gene responsible for a dominant mutant *sac51-d*, which completely suppresses the *acl5* phenotype and reverses the *acl5* transcript level without thermospermine. In *sac51-d*, one of the uORFs present in the *SAC51* mRNA contains a premature stop codon and the SAC51 protein may be overproduced, suggesting a role of thermospermine in overcoming the inhibitory effect of the uORF on the main ORF translation. Another dominant suppressor *sac52-d*, which contains a point mutation in a ribosomal protein L10 (*RPL10*) gene, has also been shown to enhance SAC51 translation and reverse the *acl5* transcript level (Imai et al. 2008).

*Arabidopsis ACL5/TSPMS* expression is enhanced by auxin, a plant hormone essential for growth (Hanzawa et al. 2000). Auxin also plays a key role in the onset of vascular formation. In *Arabidopsis*, the auxin signaling for procambial cell initiation is triggered by activation of one of auxin response factors (ARFs), MONOPTEROS (MP)/ARF5 (Hardtke and Berleth 1998). Although the promoter region of ACL5 contains a putative auxin-responsive element (AuxRE), which is targeted by ARFs (Hanzawa et al. 2000), it remains to be determined whether ARF5/MP or other ARFs bind to this element. There is increasing evidence showing that thermospermine modulates auxin signaling by locally or temporarily altering auxin flow and negatively regulates xylem development (Yoshimoto et al. 2012a, b; Tong et al. 2014). In poplar, overexpression of an *ACL5* homologue has a negative effect on auxin accumulation and expression of the class III homeodomain leucine zipper (HD-Zip III) transcription factor gene *PttHB8* (Milhinhos et al. 2013). Its *Arabidopsis* homologue *ATHB8* is induced by auxin and plays a critical role in procambial cell differentiation (Baima et al. 2001). The fact that upregulation of *PttHB8* enhances poplar *ACL5* expression suggests that HD-Zip III transcription factors positively regulate *ACL5* expression (Milhinhos et al. 2013). Taken altogether, it is suggested that auxin-induced thermospermine synthesis is integrated in a negative feedback loop controlling proliferation of cells that are destined to become xylem vessels and to die in higher plants (Takano et al. 2012; Yoshimoto et al. 2012b; Tong et al. 2014). However, as this specified function of thermospermine should be adapted only to vascular plants, the function of thermospermine in nonvascular plants such as mosses and algae remains an open question.

Although less severe than *acl5*, the knockout mutant of *SAMDC4* also exhibits a dwarf phenotype (Ge et al. 2006). Among four genes for AdoMetDC/SAMDC in *Arabidopsis*, only *SAMDC4* shows an expression pattern similar to that of *ACL5*, that is, preferential expression in vascular cells, upregulation by auxin (Cui et al. 2010), and downregulation by thermospermine (Kakehi et al. 2008). Moreover, the dwarf phenotype of *samdc4* is suppressed in *sac51-d samdc4* and *sac52-d samdc4* double mutants (Tong, unpublished data) (Fig. 3.4). These results suggest that

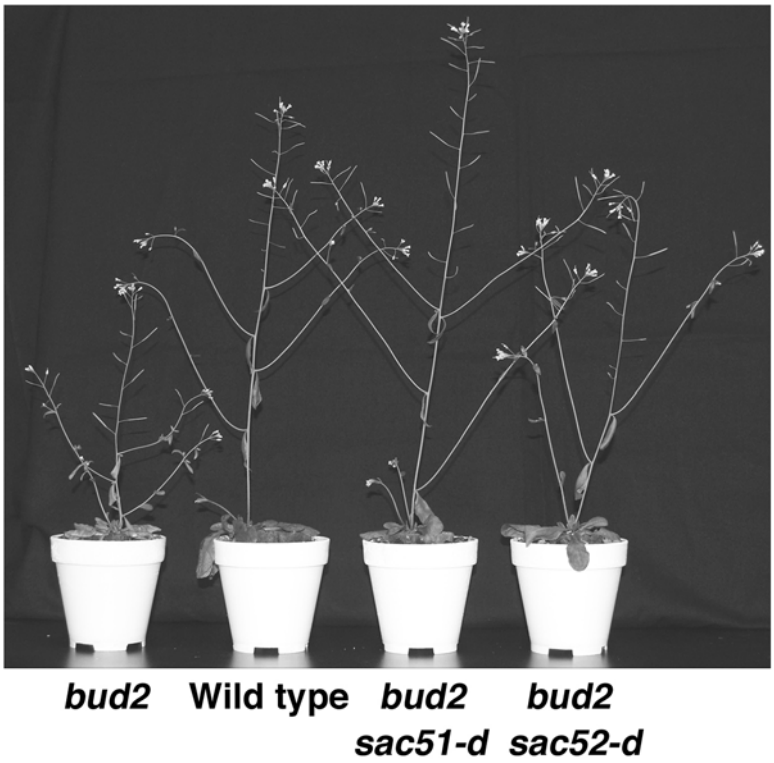


**Fig. 3.4** Forty-day-old plants of the wild type (Col-0) and *bud2, bud2 sac51-d*, and *bud2 sac52-d* mutants of *Arabidopsis* The semi-dwarf phenotype of *bud2*, a knockout mutant of *SAMDC4*, is suppressed by dominant suppressors of *acl5*, *sac51-d*, and *sac52-d*

*SAMDC4* is specialized for the synthesis of thermospermine. In contrast to the other three *SAMDC* genes, *SAMDC4* has no uORF and no intron, suggesting its distinct evolutionary origin. Further phylogenetic studies are needed to clarify the relationship between AdoMetDC/SAMDC and each aminopropyl transferase.

## 3.6 Cadaverine Generated by Lysine Decarboxylation

Cadaverine, another diamine detected in many organisms, is generated from lysine through the action of lysine decarboxylase (LDC; Fig. 3.1). Although known as a catabolite produced by protein hydrolysis during putrefaction in animal tissues, cadaverine occurs in the plant families Leguminosae, including soybean and chickpea (Lin et al. 1984; Torrigiani and Scoccianti 1995), and Solanaceae, including tobacco and tomato (Bagni et al. 1986). In these plants, cadaverine is an important intermediate in the biosynthesis of lysine-derived alkaloids such as piperidine, quinolizidine, indolizidine, and lycopodium alkaloids. Although plant LDCs form a

phylogenetically different subclade from the ODC family, recombinant LDCs from plants of Leguminosae have been shown to catalyze the decarboxylation of both lysine and ornithine (Bunsupa et al. 2012). Plant LDCs are localized in chloroplasts. In the embryonic axes of soybean, cadaverine content markedly increases immediately after germination and has been implicated in normal root development (Gamarnik and Frydman 1991).

## 3.7 Uncommon and Longer Polyamines

Uncommon and longer polyamines are present in some plant species. Norspermidine and norspermine were detected in alfalfa (Rodriguez-Garay et al. 1989) and maize (Koc et al. 1998), and the occurrence of these polyamines has suggested the presence of 1,3-diaminopropane as a precursor and the involvement of nonspecific aminopropyl transferases. It is now clear that diaminopropane is produced through oxidation of spermidine and spermine by a terminal catabolic type of polyamine oxidase (PAO) found in monocotyledonous plants (Cona et al. 2006) (see Chap. 6). 1,3-Diaminopropane is also produced as a side product of DHS and HSS reactions (Ober and Hartmann 1999) (Fig. 3.1). In prokaryotes, there are some examples of aminopropyl transferases with broad substrate specificity (Knott 2009) (see Chap. 2). SPDS with relaxed substrate specificity could explain the occurrence of norspermidine. Because norspermine can be formed by the addition of a third propylamine group to either terminus of the two propylamine groups of norspermidine, TSPMS is a more likely candidate for norspermine synthase than SPMS. Furthermore, the longer polyamines homocaldopentamine and homocaldohexamine are detected in extracts from osmotic stress-tolerant cultivars of alfalfa (Bagga et al. 1997); these can be formed successively from thermospermine. Although the possibility cannot be excluded that certain plant species possess as yet uncharacterized aminopropyl transferases for uncommon polyamines, it is possible that the substrate specificity of SPDS or TSPMS can be modulated under certain conditions. Functions of uncommon polyamines in plants remain to be addressed in future research. The dwarf phenotype of the *Arabidopsis acl5* mutant has been shown to be recovered by norspermine more efficiently than by thermospermine (Kakehi et al. 2010).

## 3.8 Conjugated Polyamines

Plant polyamines are found not only as free forms but also as perchloric acid (PCA)-soluble or PCA-insoluble conjugates with cinnamic acids such as *p*-coumaric, caffeic, and ferulic acids. Previous studies have shown that polyamine conjugates are highly accumulated by MeJA treatment, for example, in *Hyoscyamus* root cultures and tobacco thin layers (Biondi et al. 2000, 2001). These conjugates, which are known as hydroxycinnamic acid amides (HCAA) and are also termed phenolamides or phenylamides, have been implicated in promoting flowering, protecting against

pathogens, detoxifying phenolic compounds, acting as a bridge between different cell wall polymers, or serving as a reserve of polyamines that are available to actively proliferating tissues (Martin-Tanguy 1985; Bagni and Tassoni 2001; Bassard et al. 2010). The structure of spermidine-HCAA resembles microbial iron-chelating siderophores, suggesting that they act as antimicrobials and phytochelatins (Fuell et al. 2010). Genes encoding spermidine disinapoyl transferase (SDT) and spermidine dicoumaroyl transferase (SCT) were identified in *Arabidopsis* (Luo et al. 2009). *SDT* and *SCT* are highly expressed in developing embryos and root tips, respectively. In addition, an anther tapetum-specific gene encoding spermidine hydroxycinnamoyl transferase (SHT) was cloned from *Arabidopsis* (Grienenberger et al. 2009). In tobacco, genes responsible for the biosynthesis of caffeoylputrescine, dicaffeoylspermidine, and probably monoacylated spermidines are induced through the activation of the MYB8 transcription factor in response to herbivore attack (Kaur et al. 2010; Onkokesung et al. 2012).

Polyamines can be also covalently bound to glutamine residues of proteins by the action of transglutaminase (TGase). The widespread occurrence of TGase activity in all plant tissues suggests the significance of inter- or intramolecular crosslink formation of the proteins by polyamines (Serafini-Fracassini and Del Duca 2008). In chloroplasts, TGases may stabilize the photosynthetic protein complexes (Serafini-Fracassini et al. 2010). The level of conjugated polyamines and the TGase activity increase in pummelo (*Citrus grandis*) pistils in concomitance with pollen tube arrest, suggesting their involvement in self-incompatibility response (Gentile et al. 2012).

## 3.9 Conclusions

One unique feature of plant polyamines is the widespread occurrence of thermospermine. In contrast to animals, algae and lower plants such as mosses and ferns contain thermospermine but no spermine, suggesting compatible roles. However, the role of thermospermine in plant development cannot be replaced by spermine. The mode of action specific to thermospermine remains to be elucidated in future studies.

There are increasing studies suggesting the importance of covalently bound polyamines. However, the genetic research on polyamine acyltransferases and TGases is still far from complete, and further studies are certainly needed to address their functional significance. Further search for and identification of uncommon or longer polyamines might lead to medical and biotechnological benefits.

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# Part II
# Catabolism of Polyamines

# Chapter 4
# Polyamine Catabolism in Prokaryotes

**Hideyuki Suzuki and Shin Kurihara**

**Abstract** Polyamines play important roles in cell growth and proliferation. In particular, these biogenic compounds are involved in the regulation of transcription and translation processes required for bacterial proliferation. Consequently, intracellular polyamine content is strictly regulated at several levels, including biosynthesis, degradation, and uptake from and excretion into the environment. In this chapter, we discuss polyamine catabolism in prokaryotes, focusing on the well-studied polyamine catabolism pathway in *Escherichia coli. E. coli* catabolizes putrescine to succinate via γ-aminobutyraldehyde (GABA) through the aminotransferase pathway or the γ-glutamylate pathway (the Puu pathway). Excess spermidine is acetylated to yield acetylspermidine, but whether this metabolite is then excreted from cells, as it is in eukaryotes, is not clear. *Pseudomonas aeruginosa* POA1, in contrast to *E. coli*, has expanded catabolic pathways to salvage cadaverine and spermidine as carbon and nitrogen sources.

**Keywords** Aminotransferase • Putrescine utilization pathway • Spermidine acetyltransferase • Transporter • γ-Aminobutyric acid • γ-Glutamyl intermediate • γ-Glutamylation

## 4.1 Introduction

The major polyamines in *Escherichia coli* cells are putrescine, spermidine, and cadaverine; this bacterium does not synthesize spermine. In eukaryotes, the lysine residue of the precursor of eukaryotic translation initiation factor 5A (elF5A) is hypusinated by the sequential reactions of two enzymes using spermidine,

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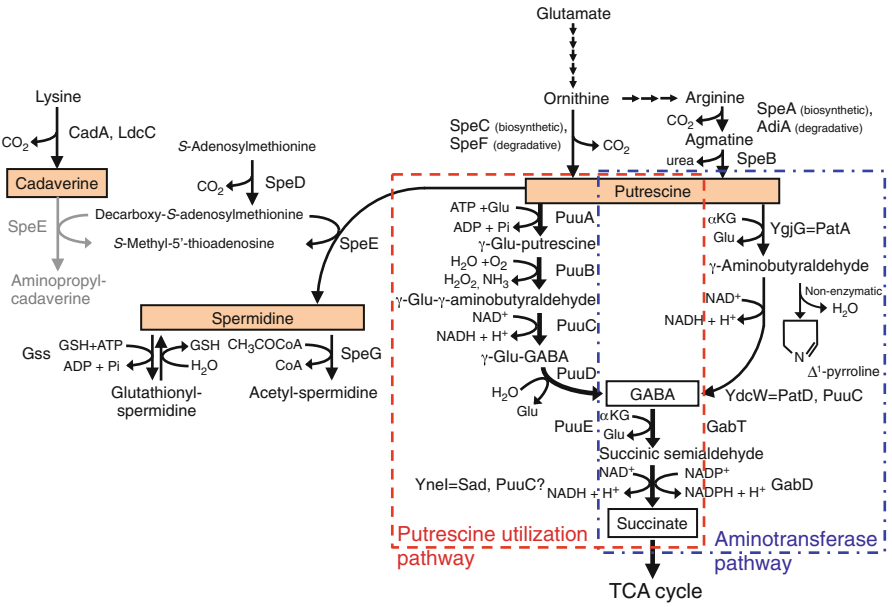


**Fig. 4.1** Metabolic map of polyamines in *Escherichia coli*

and spermidine is synthesized from putrescine (see Fig. 4.1) (Park 2006; also see Chap. 10 by Park in this book). Therefore, polyamines are essential growth factors for eukaryotic cells. In contrast, an *E. coli* strain deficient in all genes for polyamine biosynthesis can grow in polyamine-free medium in an aerobic environment, albeit at reduced growth rate (Chattopadhyay et al. 2009). However, polyamines are involved in various proliferation-associated processes in bacteria, including regulation of transcription and translation, and their intracellular levels are strictly regulated by biosynthesis, degradation, and uptake from and excretion into the environment (Igarashi and Kashiwagi 2010).

*E. coli* synthesizes putrescine from ornithine by ornithine decarboxylases (SpeC or SpeF) or from arginine by the sequential reactions of arginine decarboxylase (SpeA) and agmatinase (SpeB) (Tabor and Tabor 1985). In addition to SpeB, the *Pseudomonas aeruginosa* PAO1 strain can convert agmatine to putrescine via *N*-carbamoylputrescine by the sequential reaction of agmatine deaminase (AguA) and *N*-carbamoylputrescine amidohydrolase (AguB) (Nakada et al. 2001). Spermidine is synthesized by the addition of propylamine to putrescine by spermidine synthetase (SpeE). Cadaverine is synthesized from lysine by lysine decarboxylases (CadA or LdcC). In spermidine deficiency, relatively large amounts of aminopropylcadaverine are synthesized as a compensatory polyamine, achieved by the addition of propylamine to cadaverine, and the resulting molecule has the same effect on polypeptide synthesis and cell growth as spermidine (Igarashi et al. 1986). An in vitro study revealed that the transfer of propylamine to cadaverine is mediated

by SpeE (Bowman et al. 1973). Hanfery et al. (2011) showed that *Campylobacter jejuni* has an alternative biosynthetic pathway of spermidine. In that pathway, not propylamine but carboxypropylamine is added from aspartate β-semialdehyde to putrescine to yield carboxyspermidine by carboxyspermidine dehydrogenase, followed by decarboxylation to yield spermidine by carboxyspermidine decarboxylase.

In this chapter, we discuss various bacterial polyamine catabolic pathways, focusing mainly on those of *E. coli*, and describe how polyamine production may be regulated and used as a bacterial feedstuff.

## 4.2 Putrescine Catabolic Pathways in *E. coli*

Two pathways, the aminotransferase pathway and the putrescine utilization pathway (the Puu pathway) (see Fig. 4.1), are responsible for the catabolism of putrescine to yield nitrogen and carbon sources for growth.

### *4.2.1 Aminotransferase Pathway*

The aminotransferase pathway was first reported by Shaibe et al. (1985a, b) as a component of the arginine and ornithine catabolic networks (see Fig. 4.1). In this pathway, either arginine or ornithine is catabolized to succinate as a sole N source via putrescine and γ-aminobutyric acid (GABA). During the process, one of the amino groups of putrescine is transferred to α-ketoglutarate by putrescine aminotransferase (PatA=YgjG) to form γ-aminobutyraldehyde and glutamate. γ-Aminobutyraldehyde is further oxidized to GABA by γ-aminobutyraldehyde dehydrogenase (PatD=YdcW). The amino group of GABA is transferred to α-ketoglutarate by GABA aminotransferase (GabT) to form succinic semialdehyde and glutamate. Then, succinic semialdehyde is oxidized to succinate by succinic semialdehyde dehydrogenase (GabD). Approximate map positions of *patA* and *patD* on the genome were also reported by Shaibe et al. (1985a), although the map position of *patD* that they defined conflicts with current knowledge. PatD protein was purified in 1987 (Prieto et al. 1987). These genes, however, were not cloned until 2003 (Samsonova et al. 2003). The *ygjG* (=*patA*) gene was cloned and PatA (=YgjG) protein was purified to homogeneity from an overexpressing strain and then characterized (Samsonova et al. 2003). In the case of PatD (=YdcW), the protein was purified and the amino acid sequences of trypsin-digested peptides were compared with the *E. coli* genome database to identify its gene as *ydcW* (Samsonova et al. 2005). The $k_{cat}/K_m$ value of YdcW against γ-aminobutyraldehyde is two orders of magnitude greater than that against butyraldehyde, indicating that γ-aminobutyraldehyde is its natural substrate. The crystal structure of YdcW (=PatD) had also been solved without knowing which pathway it involves and what is its cognate substrate (Gruez et al. 2004).

GABA released by PatD (=YdcW) is further converted to succinate for entry to the TCA cycle by the sequential reactions of GABA aminotransferase (GabT) and succinic semialdehyde dehydrogenase (GabD) (Schneider et al. 2002). Strains with deletion mutations in these genes grew normally on all tested nitrogen sources with the exception of GABA. That *gabP* gene is located next to *gabT*, which encodes a GABA transporter, and that *gabDTP* forms an operon (Maciag et al. 2011) indicates that the products of *gabDTP* genes constitute a pathway for utilization of GABA as an N source. However, a Δ*gabDT* strain is able to grow on putrescine as an N source, and still retains GABA aminotransferase and succinic semialdehyde dehydrogenase activities. Furthermore, its generation time when putrescine is used as an N source is comparable to wild-type strains (Schneider et al. 2002). Thus, there must be another pathway by which GABA generated from putrescine can be catabolized. Indeed, this is the putrescine utilization pathway that is described in Sect. 4.2.3 following.

In *P. aeruginosa* PAO1, the first enzyme of the aminotransferase pathway was reported as putrescine-pyruvate aminotransferase, which generates γ-aminobutyraldehyde and L-alanine (Lu et al. 2002; Chou et al. 2013).

### *4.2.2 Regulation of the Aminotransferase Pathway*

The expression of *patA* gene is under the control of NtrC (nitrogen regulatory protein C) and $\sigma^{54}$ (Zimmer et al. 2000; Samsonova et al. 2003; Schneider et al. 2013) and is also subjected to catabolite repression (Shaibe et al. 1985b). Because loss of both $\sigma^{S}$ and $\sigma^{54}$ diminished PatA activity, *patA* is transcribed with RNA polymerase not only with $\sigma^{54}$ but also with $\sigma^{S}$ (Schneider et al. 2013). The expression of *ydcSTUVW* (*ydcW*=*patD*) operon is regulated exceptionally by Nac (nitrogen assimilation control protein) and $\sigma^{S}$ (Schneider et al. 2013).

General gene regulation by $\sigma^{S}$, $\sigma^{54}$, NtrC, and Nac can be explained as follows. In a two-component nitrogen regulatory system, the sensor histidine kinase, NtrB, senses nitrogen limitation in the medium and undergoes autophosphorylation. Then, NtrB transfers the phosphoryl group to the aspartate residue of its cognate response regulator (transcription regulator), NtrC. Phosphorylated NtrC is active, and it helps the closed complex of RNA polymerase with $\sigma^{54}$ (nitrogen limitation σ factor) at the promoter to form an open complex to promote transcription initiation of genes required under nitrogen-limiting conditions (Zimmer et al. 2000). The *nac* gene is under the control of NtrC and $\sigma^{54}$, and is expressed upon nitrogen starvation. Nac activates RNA polymerase in cooperation with $\sigma^{70}$ (housekeeping σ factor) to transcribe a number of operons whose products can supply the cell with ammonium or glutamate from alternative organic sources (Muse and Bender 1998). By contrast, $\sigma^{S}$ is recognized as the master regulator of the general stress response, which is often accompanied by reduction or cessation of growth, and provides the cells with the ability to survive the actual stress as well as additional stresses not yet encountered (Hengge-Aronis 2002).

*gabDTP* consists of an operon whose $gabD_{p2}$ promoter is regulated by $\sigma^S$ (Metzner et al. 2004; Maciag et al. 2011). Metzner showed that *csiD-ygaF-gabDTP* also consists of an operon and whose $csiD_p$ promoter depends on cAMP-CRP and $\sigma^S$; the operon is activated exclusively upon carbon starvation and onset of stationary phase (Metzner et al. 2004). In response to low nitrogen, the expression of *gabDTP* is upregulated by Nac and $\sigma^{70}$ from an alternative promoter, $gabD_{p1}$ (Schneider et al. 2002).

### 4.2.3 The Putrescine Utilization Pathway (the Puu Pathway)

The other catabolic pathway of putrescine is the putrescine utilization pathway that we first reported in 2005 (Fig. 4.1) (Kurihara et al. 2005). In this pathway, extracellular putrescine is transported into the cell by transporter PuuP (its gene, *puuP*=*ycjJ*). One of the amino groups of putrescine is γ-glutamylated by γ-glutamylputrescine synthetase (PuuA) (its gene, *puuA*=*ycjK*) using ATP to generate γ-glutamylputrescine (Kurihara et al. 2008). γ-Glutamylputrescine is oxidized to γ-glutamyl-γ-aminobutyraldehyde by PuuB (its gene, *puuB*=*ordL*), which is further oxidized to γ-glutamyl-GABA by PuuC (its gene, *puuC*=*aldH*). Then, the γ-glutamyl moiety is cleaved by γ-glutamyl-GABA hydrolase (PuuD) (its gene, *puuD*=*ycjL*) to release glutamate and GABA (Kurihara et al. 2006). The amino group of GABA is transferred to α-ketoglutarate by GABA aminotransferase (PuuE) (its gene, *puuE*=*goaG*) to generate succinic semialdehyde. Then, succinic semialdehyde is oxidized to succinate by succinic semialdehyde dehydrogenase (YneI=Sad) (its gene, *yneI*=*sad*) (Kurihara et al. 2010).

Sad activity was first reported as the second succinic semialdehyde dehydrogenase, which is smaller than GabD and prefers $NAD^+$ as a cofactor, whereas GabD prefers $NADP^+$ (Donnelly and Cooper 1981). The Sad is induced by succinic semialdehyde, whereas *gabD* is induced by GABA coordinately with *gabT* (Donnelly and Cooper 1981). More than 25 years after that report, the gene coding Sad was first identified as *yneI* (Fuhrer et al. 2007).

PuuC was originally identified as γ-glutamyl-γ-aminobutylaldehyde dehydrogenase of the Puu pathway (Kurihara et al. 2005), but thereafter Schneider and Reitzer (2012) showed that PuuC has broad substrate specificity and utilizes not only γ-glutamyl-γ-aminobutyraldehyde, but also γ-aminobutyraldehyde and succinic semialdehyde, as substrates by comparing the activities of cell-free extracts of strains with various combinations of mutations. They also showed that PatD could be replaced by PuuC in vivo to support the growth of cells with putrescine as a sole N source. However, whether PuuC has sufficient succinic semialdehyde dehydrogenase activity in vivo to support the growth of cells with putrescine as a sole C source instead of GabD was not shown. Because *puu* genes exist as a gene cluster, it is quite likely that the natural substrate of PuuC is γ-glutamyl-γ-aminobutyraldehyde. The Δ*gabD* Δ*yneI* *aldA*$^+$ *puuC*$^+$ strain did not grow on a M9 putrescine-ammonium

chloride plate, which contains putrescine as a sole carbon source, although the Δ*gabD yneI*$^+$ *aldA*$^+$ *puuC*$^+$ strain grew on this plate at 20 °C (Kurihara et al. 2010). This observation clearly indicates that YneI, but not PuuC, supports the growth of the cells using putrescine as a sole carbon source in the absence of GabD. Although both *puu* genes and *yneI* are induced by putrescine (Kurihara et al. 2010), they are located separately on the genome at 29.3 min (Kurihara et al. 2005) and 34.7 min (Fuhrer et al. 2007), respectively.

It should be emphasized that an amino group is very reactive and one of the two amino groups of putrescine is first protected with a γ-glutamyl moiety by the reaction of PuuA at the expense of ATP before the second amino group is oxidized. Then, the γ-glutamyl moiety of γ-glutamyl-GABA is cleaved by PuuD to release GABA; the newly released amino group is further catabolized by GABA aminotransferase, PuuE. In fact, γ-aminobutyraldehyde, an intermediate of the aminotransferase pathway, is unstable, and its amino and aldehyde groups tend to form cyclic $\Delta^1$-pyrroline nonenzymatically (Shaibe et al. 1985a). The γ-glutamylation and de-γ-glutamylation of the reactive amino group are exactly the same processes as the protection and deprotection of reactive groups performed during the chemical synthesis of some compounds. This is a rare example of the physiological role of γ-glutamylation, although there are some other examples (de Azevedo Wasch et al. 2002; Yao et al. 2011). PuuA catalyzes γ-glutamylation of putrescine to form the γ-glutamyl linkage of γ-glutamylputrescine, whereas PuuD catalyzes hydrolysis of the γ-glutamyl linkage of γ-glutamyl-GABA. If γ-glutamylputrescine, and not γ-glutamyl-GABA, were the preferred substrate of PuuD, there would be no rationale for PuuA to synthesize γ-glutamylputrescine at the expense of ATP. $K_m$ values against γ-glutamylputrescine and γ-glutamyl-GABA were 18.5 and 2.93 mM, respectively. Also, $k_{cat}/K_m$ values against γ-glutamylputrescine and γ-glutamyl-GABA were 23.5 and 850, respectively (Kurihara et al. 2005). These results indicate that γ-glutamyl-GABA is a far better substrate for PuuD than is γ-glutamylputrescine.

### *4.2.4 Regulation of the Puu Pathway*

Genes coding for the Puu pathway make a gene cluster, *puuPADRCBE* (Fig. 4.2) (Kurihara et al. 2005). *puuA*, *B*, *C*, *D*, *E*, and *P* code for the members of the Puu pathway, as described in Sect. 4.2.3, and *puuR* codes for the repressor (PuuR) of *puuAP* and *puuDRCBE* operons. There are four promoters in this gene cluster (Fig. 4.2): three of them are reported to be $\sigma^S$ dependent (Reitzer and Schneider 2001; Maciag et al. 2011) and the other is NtrC-$\sigma^{54}$ dependent (Reitzer and Schneider 2001; Zhao et al. 2010). Maciag et al. (2011) suggested that $\sigma^S$ regulates the expression of genes from arginine to succinate via putrescine through the Puu pathway.

Both *puu* genes and *yneI* are induced by putrescine (Kurihara et al. 2010). Although *puu* genes are regulated by the repressor PuuR (Kurihara et al. 2005, 2008, 2009, 2010; Nemoto et al. 2012), *yneI* is PuuR independent (Kurihara et al. 2010).

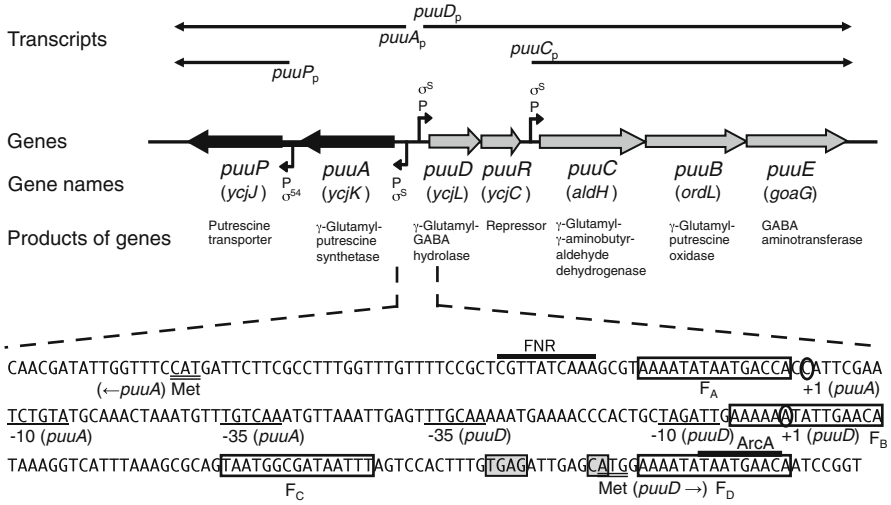


**Fig. 4.2** Putrescine pathway (Puu) genes cluster of *Escherichia coli*, their transcripts, and postulated recognition sites by regulatory proteins in *puuA–puuD* intergenic region. The evidence of the transcripts and their regulation by different σ factors is described in the text. Inside the *parentheses* are the previously assigned gene names. DNA sequence shows the intergenic region and the regulatory sequences between *puuA* and *puuD*. The nucleotide sequence from position −210 to +90 from the *puuD* transcription start site is shown. The transcriptional start sites (+1) are *circled*, and the inferred −10 and −35 hexamers of the *puuA* and *puuD* promoters are *underlined*. The initiation codons of the *puuA* and *puuD* are also *double underlined* with the label "Met." The 15-bp PuuR-binding motifs are boxed and labeled $F_A$, $F_B$, $F_C$, and $F_D$, which were determined by DNase I footprint assay (Nemoto et al. 2012). A cAMP-CRP recognition site (Shimada et al. 2011) is *boxed gray*. Predicted FNR and ArcA recognition sites (Partridge et al. 2006) are shown by *thick black lines* above the sequence

Schneider and Reitzer (2012) showed that the in-frame Δ*puuA* strain, which does not cause a polar effect on *puuP*, could grow on putrescine as an N source if the enzymes of the aminotransferase pathway were present. By contrast, they revealed that Δ*puuP* strain could not grow on putrescine as an N source even though the enzymes of aminotransferase pathway are present. This finding conflicts with our result that Δ*puuA*::*kan* strain, which could not utilize putrescine as a sole C source and a sole N source, complemented the growth on putrescine with a plasmid that expresses only PuuA (Kurihara et al. 2008). PuuP is the essential putrescine transporter required for the cells to grow on putrescine as an N source (Kurihara et al. 2009; see Chap. 14 by Kurihara and Suzuki in this book). Because the *puuP* gene can also be transcribed under the control of NtrC and $\sigma^{54}$ from the promoter $puuP_p$, which is separate from the promoter $puuA_p$ under the control of $\sigma^S$, its expression could be coordinated with that of the aminotransferase pathway.

We observed that the expression of *puu* genes was induced at high aeration by putrescine and reduced by either glucose, succinate, or $NH_4Cl$ (Kurihara et al. 2005, 2006). Since then, the molecular mechanisms of regulations of *puu* genes have been elucidated. The expression of *puu* genes is repressed by PuuR, which has a

helix-turn-helix DNA-binding motif and is coded in the *puu* gene cluster; this cluster is induced in the presence of putrescine (Nemoto et al. 2012). A probe corresponding to the intergenic region between *puuA-puuD* was shifted in the presence of purified $His_6$-PuuR by a gel mobility shift assay, and the amount of the shifted bands was inversely proportional to the concentration of putrescine but not to that of spermidine. However, the probes corresponding to the intergenic regions between *puuA-puuP* and *puuR-puuC* were not shifted. Transcript sizes were checked, and it was confirmed that *puuAP* and *puuDRCBE* transcripts exist. DNase I footprint analysis of the intergenic region between *puuA-puuD* showed there are four PuuR-binding sites (Fig. 4.2), and Nemoto et al. (2012) proposed a consensus binding sequence that consists of 15 nucleotides with an asymmetrical recognition sequence, AAAATATAATGAACA, which is in the PuuR-binding site from 20 nucleotides (ATGGaCAATATATTGaCCAT) with an inverted repeated symmetry that was suggested by the curator of the Regulon Database (URL: http://regulondb.ccg.unam.mx/).

*puu* genes are repressed by the addition of glucose, and the regulatory mechanisms involved are now known. There is a cAMP-CRP recognition site between the transcription initiation site and the initiation codon of *puuD* (Fig. 4.2) (Shimada et al. 2011). Terui et al. (2014) showed that the expression of *puuR* from the $puuD_p$ promoter is positively regulated by glucose. That is, in the presence of glucose the concentration of cAMP decreases and CRP no longer binds to the cAMP–CRP recognition site in front of the initiation codon of *puuD*. This stage releases the inhibition of transcription of *puuR*, and PuuR as synthesized then represses transcription from $puuA_p$ and $puuD_p$ as a result.

Partridge et al. (2006) observed that *puu* genes are induced upon a shift from anaerobic to aerobic conditions in *E. coli*. They predicted an FNR recognition site and an ArcA recognition site in the *puuA–puuD* intergenic region and suggested the de-repression of the *puu* genes in the presence of $O_2$. They also showed that the supercoiling of plasmid was changed along with the shift from anaerobic to aerobic condition, but this phenomenon was delayed in the *puuA* mutant. This investigation leads to a model in which $O_2$ induces the Puu pathway, reduces the intracellular putrescine concentration, and causes the topological changes in DNA that influence the transcription of various genes.

Another possible regulatory mechanism of the Puu pathway is the metal-catalyzed oxidative modification of PuuA followed by proteolytic degradation. This regulatory mechanism was proposed to be one of the regulatory mechanisms of glutamine synthetase, a key enzyme of ammonium assimilation (Liaw et al. 1993). PuuA has high amino acid sequence similarity to glutamine synthetase. The enzymatic reactions catalyzed by glutamine synthetase and PuuA are also very similar in terms of the amide bond formation between the γ-carboxyl group of glutamate and ammonia, and the γ-carboxyl group of glutamate and the amino group of putrescine, respectively. Both enzymes exist as homododecamers and require $Mg^{2+}$ or $Mn^{2+}$ for activity (Stadtman and Ginsburg 1974; Kurihara et al. 2008). The important amino acid residues for two metal-binding sites, the glutamate and ATP-binding sites, are well conserved. It was reported that the oxidative modification of His-269 to Asn and Arg-344

to Gln induces the loss of activity of glutamine synthetase followed by increased susceptibility to proteolytic degradation (Liaw et al. 1993). Because these residues are also conserved in PuuA in addition to the similarities already mentioned, it is plausible that PuuA is also subjected to metal-catalyzed oxidative modification followed by proteolytic degradation. In fact, PuuA protein is much more unstable than many other proteins. Because PuuA is the key enzyme of the Puu pathway and the catabolism of putrescine, it is quite rational that its prompt decay allows *E. coli* to adapt to the sudden decrease of intracellular putrescine concentration.

## 4.3 Catabolic Pathways of Spermidine

*E. coli* can increase intracellular spermidine concentration by synthesizing it from putrescine or by importing it from the environment by PotABCD (Igarashi and Kashiwagi 1999). Conversely, intracellular spermidine concentration is reduced by the activity of spermidine acetyltransferase. This enzyme acetylates spermidine to form acetylspermidine by using acetyl-CoA (Fukuchi et al. 1994). In eukaryotes acetylspermidine is excreted from the cell (Gerner and Meyskens 2004), but the fate of acetylspermidine in *E. coli* is not clear. Intracellular spermidine concentration in the wild-type strains does not vary dramatically between cells grown in the presence and absence of 0.5 mM spermidine. On the other hand, the spermidine acetyltransferase-deficient (*speG*$^-$) strain markedly accumulates spermidine when it is grown in the presence of 0.5 mM spermidine, but not in the absence of spermidine (Fukuchi et al. 1995). The growth of the *speG*$^-$ strain in M9 medium was normal in the presence and absence of 0.5 mM spermidine, but its viability at the late stationary phase was greatly decreased compare to the wild type. Fukuchi et al. (1995) suggested that a decrease of various protein syntheses that included ribosome modulation factor essential for cell viability at the stationary phase was caused by the accumulation of spermidine in the *speG*$^-$ strain.

The other enzyme that may decrease the intracellular spermidine in *E. coli* is glutathionylspermidine synthetase/amidase (Gss = Gsp). Gss was first studied extensively by Bollinger et al., who found that the enzyme is bifunctional and consists of two domains that are responsible for catalysis of the reverse reactions (Bollinger et al. 1995; Kwon et al. 1997). The three-dimensional structure of Gss has been reported (Pai et al. 2006), and its role in redox regulation was studied (Chiang et al. 2010). However, there has been no report if it regulates the intracellular spermidine concentration.

*P. aeruginosa* PAO1 has spermidine dehydrogenase (SpdH), which cleaves spermidine into 1,3-diaminopropane and γ-aminobutyraldehyde and spermine into spermidine and 3-aminopropanaldehyde. γ-Aminobutyraldehyde is further oxidized to GABA by KauB, which corresponds to PatD, followed by catabolism to succinate by GabT and GabD. 3-Aminopropanaldehyde is oxidized to β-alanine by KauB, and subsequently catabolized to acetyl-CoA by β-alanine-pyruvate aminotransferase (BauA) and malonic semialdehyde dehydrogenase (BauB). However,

SpdH is not induced by exogenous polyamines, and the *spdH* knockout mutant grows normally on spermidine and spermine (Dasu et al. 2006). According to a database search using Blastp (Altschul et al. 1997), *E. coli* does not have a SpdH homologue. In *P. aeruginosa* PAO1, spermidine is catabolized by the *pau* pathway, which is discussed in the next section, and all seven *pauA* genes-deficient mutants cannot grow on spermidine.

## 4.4 Catabolic Pathways of Cadaverine

In *E. coli* a cadaverine-specific catabolic pathway has not yet been reported. Despite the lack of in vivo evidence, the activity of PatA (=YgjG) toward cadaverine is comparable to that toward putrescine (Samsonova et al. 2003), and the activity of PuuA toward cadaverine is about one third of that toward putrescine in vitro, implying that both the aminotransferase and the Puu pathways are involved in cadaverine catabolism. If this is true, then either PuuE or GabT might also use δ-aminovalerate, and either YneI or GabD could use glutamic semialdehyde as substrate. However, this requires further experimental testing. In *P. aeruginosa* PAO1, seven *pauA* genes, four *pauB* genes, one *pauC* gene, and two *pauD* genes, which correspond to *puuA, B, C,* and *D* gene of the Puu pathway, respectively, are responsible to various polyamines catabolism. Each PauA has different specificity toward each polyamine, and specific combination of *pauA* knockouts is required to abolish the utilization of specific polyamines (Yao et al. 2011). It has also been demonstrated that PauR controls *pau* promoters in response to putrescine and cadaverine (Chou et al. 2013).

In this strain, spermidine-inducible genes overlap almost completely with putrescine-inducible *pau* genes, with the exception of *pauA3B2* and *bauABCD* operons (Yao et al. 2011). PauA3 and PauB2 are involved in the catabolism of diaminopropane, generated from the aminopropyl moiety of spermidine, and BauA and BauB are involved in β-alanine catabolism as described in Sect. 4.3 (Yao et al. 2011). A single knockout mutation of the *pauA2* gene blocks growth on spermidine completely, but the mutant can grow on putrescine, cadaverine, or diaminopropane (Yao et al. 2011). This indicates that PauA2 is a spermidine-specific γ-glutamyl ligase. However, it is still unclear whether the amino group of the aminopropyl moiety or that of the aminobutyl moiety is γ-glutamylated and how the internal C–N bond is cleaved.

## 4.5 Future Perspectives

Can *E. coli* degrade cadaverine and spermidine as does *P. aeruginosa*? How does *P. aeruginosa* degrade spermidine through the Pau pathway? How does the intracellular putrescine concentration respond to various stresses? These are questions that should be answered in the near future.

Global warming has become a major issue, and there is a move away from fossil resources toward recyclable resources, not only as energy sources but also as chemical feedstocks. Polyamines are used as intermediate materials in the production of synthetic fibers and fabrics. At present, they are mainly produced from petroleum by industrial chemical processes, but putrescine, spermidine, and cadaverine can all be synthesized biologically from arginine, ornithine, and lysine. These amino acids are produced industrially by fermentation. To obtain a higher polyamine yield and industrialize the process, we await a more detailed understanding of polyamine metabolism in bacteria.

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# Chapter 5
# Mammalian Polyamine Catabolism

**Tracy Murray-Stewart and Robert A. Casero Jr.**

**Abstract** Intracellular mammalian polyamine catabolism occurs through two distinct pathways, both of which culminate in oxidation reactions that generate highly reactive, potentially toxic by-products. In the back-conversion to spermidine, spermine can either undergo direct oxidation by spermine oxidase (SMOX) or be acetylated by spermidine/spermine $N^1$-acetyltransferase (SSAT), followed by subsequent oxidation by acetylpolyamine oxidase (APAO). Spermidine undergoes acetylation and oxidation back to putrescine through this same SSAT/APAO pathway. Polyamines are absolutely essential for cell viability and proliferation, and polyamine biosynthesis and intracellular concentrations are frequently upregulated in hyperproliferative conditions such as cancer. As a result, many studies have successfully focused on the induction of polyamine catabolism as a rational target for antiproliferative chemotherapeutic intervention. However, it is also becoming apparent that chronically elevated levels of polyamine catabolism in nontumorigenic cells can have disease implications. A variety of stimuli, including microbial pathogens, inflammatory signals, and tissue injury, have now been identified to induce the polyamine catabolic enzymes. In addition to the back-conversion of polyamines, these reactions also release the reactive oxygen species precursor hydrogen peroxide as well as potentially toxic aldehydes. These metabolites as well as the reduction in spermine and spermidine levels can have deleterious physiological effects resulting in the manifestation and promotion of multiple pathologies. This chapter focuses on recent discoveries in the regulation of the mammalian polyamine catabolic enzymes and the pathophysiological effects of this upregulation.

**Keywords** Aldehyde • Epigenetic • Inflammation • Reactive oxygen species • Spermidine/spermine $N^1$-acetyltransferase • Spermine oxidase

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## 5.1 Introduction

Putrescine, spermidine, and spermine constitute the naturally occurring mammalian polyamines. As in all cells, the mammalian polyamines are absolutely essential for viability through their contributions to critical cellular functions, including nucleic acid and protein synthesis, transcriptional and translational regulation, and macromolecular structural integrity (Pegg 1988, 2009; Saini et al. 2009; Park et al. 2010). Spermine, in particular, has also been shown to provide significant protection against oxidative damage (Ha et al. 1998a, b; Rider et al. 2007). For these functional interactions, polyamine homeostasis must be tightly regulated: an excess of intracellular polyamines becomes rapidly toxic (Tabor and Rosenthal 1956), whereas highly upregulated polyamine catabolism reduces the natural intracellular polyamines and generates toxic by-products (Wang and Casero 2006).

## 5.2 The Mammalian Polyamine Catabolic Enzymes and Their Metabolites

In mammalian cells, the catabolism of spermine to spermidine occurs via one of two distinct pathways. As a substrate for spermidine/spermine $N^1$-acetyltransferase (SSAT), spermine can be converted to $N^1$-acetylspermine, which is subsequently oxidized by the FAD-dependent acetylpolyamine oxidase (APAO) to form spermidine. Conversely, spermine can be directly oxidized by spermine oxidase (SMOX) to form spermidine. Spermidine is then back-converted to putrescine through the two-step SSAT/APAO reaction that includes an $N^1$-acetylspermidine intermediate (Fig. 5.1).

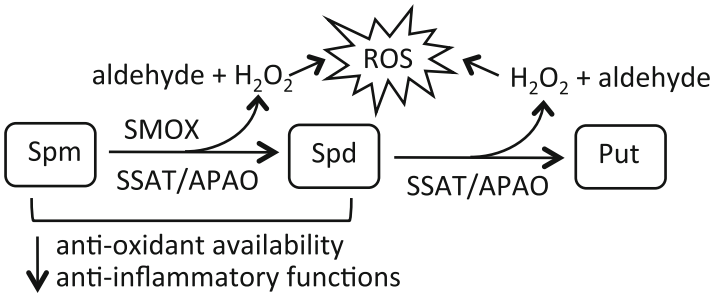


**Fig. 5.1** The mammalian polyamine catabolic pathway. Spermine (*Spm*) is back-converted to spermidine (*Spd*) by either spermine oxidase (*SMOX*) or spermidine/spermine $N^1$-acetyltransferase (*SSAT*) followed by acetylpolyamine oxidase (*APAO*). Spermidine is further back-converted to putrescine (*Put*) through the same SSAT/APAO mechanism. Both oxidation reactions generate the reactive oxygen species (*ROS*) precursor $H_2O_2$ and aldehydes as by-products. The resulting reduction in spermine and spermidine pools implies diminished antioxidant and antiinflammatory functions

### 5.2.1 *Spermidine/Spermine $N^1$-Acetyltransferase and $N^1$-Acetylpolyamine Oxidase*

SSAT is the rate-limiting enzyme of the polyamine catabolic pathway that catalyzes the transfer of an acetyl group from acetyl coenzyme A to the $N^1$ position of spermine or spermidine (Casero and Pegg 1993; Pegg 2008; Matsui et al. 1981). The resulting molecule has a reduced positive charge that alters its binding affinity for cellular macromolecules and facilitates its export from the cell. Additionally, $N^1$-acetylated spermine or spermidine can be oxidized by APAO, resulting in spermidine or putrescine, respectively, hydrogen peroxide ($H_2O_2$), and 3-acetamidopropanal (Holtta 1977; Wu et al. 2003; Wang et al. 2005b). $H_2O_2$ is a potential reactive oxygen species (ROS); however, in most cases, the peroxisomal localization of the APAO enzyme appears to protect the cell from its oxidative effects.

### 5.2.2 *Spermine Oxidase*

The *SMOX* gene encoding spermine oxidase is alternatively spliced, and multiple isoforms have been characterized in both human and mouse (Wang et al. 2001; Murray-Stewart et al. 2002, 2008; Cervelli et al. 2003). The catalytically active SMOX isoforms are FAD-dependent enzymes that directly oxidize spermine to yield spermidine, $H_2O_2$, and the aldehyde 3-aminopropanal. Importantly, these isoforms are found in significant amounts in both the cytoplasm and nucleus, resulting in the production of $H_2O_2$ as an ROS precursor in close proximity to DNA and chromatin while catalyzing the oxidation of the free-radical scavenger spermine (Murray-Stewart et al. 2008; Cervelli et al. 2004; Bianchi et al. 2005). SMOX activity therefore has the potential to significantly contribute to cellular oxidative damage and subsequent disease development. Additionally, the 3-aminopropanal produced through SMOX activity can be further metabolized to form acrolein, which also has toxic cellular effects and physiological implications.

## 5.3 Induction of Polyamine Catabolism

Regulation of SSAT occurs at nearly every level from transcription through protein stabilization, resulting in an enzyme that is highly inducible (Casero and Pegg 1993; Pegg 2008). Elevated levels of the natural polyamines themselves stimulate SSAT induction, and polyamine analogues have been extensively studied for their ability to highly induce SSAT activity in tumor cells as a chemotherapeutic strategy (Nowotarski et al. 2013; Casero and Marton 2007). Other stimuli for SSAT induction include certain proinflammatory cytokines, nonsteroidal antiinflammatory drugs (NSAIDs), hormones, stress, and common cytotoxic drugs such as cisplatin (Casero and Pegg 2009). As the acetylated polyamine substrates for oxidation by

APAO are produced only through SSAT activity, APAO is in general constitutively expressed and rate limited by SSAT.

In contrast to APAO, oxidation of spermine via SMOX is inducible; however, most of its regulation appears to be at the level of transcription (Wang et al. 2005a). Similar to SSAT, SMOX can be induced by polyamine analogues and certain pro-inflammatory cytokines, leading to oxidative DNA damage. SMOX is also induced upon microbial pathogen infection, in states of chronic inflammation, and following tissue injury (Casero and Pegg 2009). Recent advances in the regulation and induction of the polyamine catabolic enzymes and their pathophysiological implications are discussed in the text that follows.

### *5.3.1 Epigenetic Regulation of Mammalian Polyamine Catabolism*

Epigenetics refers to heritable alterations in gene expression that are not the result of changes in nucleotide sequence. Mechanisms that induce these changes include posttranslational modifications of histone proteins, such as acetylation, methylation, and phosphorylation, and the methylation of CpG dinucleotides. Few studies have focused on the direct epigenetic regulation of the polyamine catabolic enzymes, although the promoter regions of both *SAT1* and *SMOX* genes contain identifiable CpG islands, suggesting the potential for transcriptional regulation through DNA methylation. The influence of methylation on SSAT expression was initially identified in lung cancer cell lines derived from female patients. The *SAT1* gene is located on the X chromosome; therefore, the female cell lines expressed variable levels of basal SSAT expression and responsiveness to polyamine analogues that correlated with the expression of one or both alleles, and this expression was regulated by DNA methylation (Mank-Seymour et al. 1998). Recent studies in the prefrontal cortex have also correlated *SAT1* promoter DNA CpG hypermethylation with decreased SSAT mRNA expression (Fiori and Turecki 2011). As DNA methylation is often increased in cancer, the downregulation of polyamine catabolic enzymes via DNA hypermethylation could provide a mechanism for maintaining the high levels of polyamines required for tumor proliferation.

SSAT expression is also indirectly regulated through epigenetic mechanisms. The *SAT1* gene promoter contains a polyamine-responsive element (PRE) that enables transcriptional activation via the binding of NRF2 and polyamine-modulating factor 1 (PMF1) (Wang et al. 1998, 1999). In human non-small cell lung cancer cell lines that respond to polyamine analogue treatment with a large induction of SSAT, NRF2 is constitutively bound at the PRE by alterations of the KEAP1 protein, which normally sequesters it in the cytoplasm (Singh et al. 2006; Itoh et al. 1999). Recent studies have shown that NRF2-dependent transcriptional regulation of SSAT is influenced by the histone acetylation status of a specific microRNA, miR-200a, which targets the 3′-UTR of the KEAP1 mRNA. Treatment of polyamine analogue-resistant small cell lung cancer cells with a histone deacetylase (HDAC) inhibitor increased expression of miR-200a, which subsequently down-regulated KEAP1 mRNA and protein and allowed the translocation of NRF2 to the

nucleus. NRF2 occupancy was enriched at the PRE of the SSAT promoter, resulting in the sensitization of these phenotypically resistant cells to the antiproliferative effects of SSAT upregulation by the bis(ethyl)polyamine analogues (Murray-Stewart et al. 2013). These data suggest the use of HDAC inhibitors in combination with SSAT-inducing polyamine analogues as an effective chemotherapeutic strategy in patients harboring clinically aggressive small-cell lung cancers.

Last, PMF1, which responds to increased polyamines and their analogues through NRF2-dependent transcriptional activation of *SAT1*, is frequently hypermethylated and silenced in human bladder tumors (Aleman et al. 2008). The degree of PMF1 methylation and the corresponding loss of PMF1 expression is significantly correlated with increased tumor stage and grade and is prognostic of poor overall survival. Furthermore, PMF1 hypermethylation is detectable in urinary specimens and can accurately distinguish bladder cancer patients from controls; a subsequent study indicated PMF1 methylation status as a predictor for patient response to a common bladder tumor therapy (Alvarez-Mugica et al. 2013). These data indicate a role for PMF1-mediated induction of SSAT in blocking the progression of bladder cancer, perhaps by maintaining intracellular polyamine levels below those necessary for tumor proliferation. Unfortunately, the downstream effects of PMF1 silencing on polyamine catabolism have not been reported.

### *5.3.2 Regulation by Infectious/Inflammatory Agents*

The upregulation of polyamine catabolism in response to microbial infection and inflammatory stimulus is becoming a common theme. Several bacterial pathogens have now been identified to induce SMOX expression in host cells, the ultimate outcome of which is oxidative DNA damage, apoptosis, and an increased potential for neoplastic transformation. These pathogens include *Helicobacter pylori* and the enterotoxigenic *Bacteroides fragilis*, both of which are described in greater detail in the sections that follow (Xu et al. 2004; Goodwin et al. 2011). Additionally, a constituent of gram-negative bacterial cell walls, endotoxin (LPS), induces the transcription of both SSAT and SMOX in the kidney (Zahedi et al. 2010), and expression of an HIV1-encoded protein induces SMOX in neuronal cells (Capone et al. 2013).

Polyamine catabolism contributes to inflammation through the production of ROS; however, it is also regulated as a result of inflammatory signals. Both SMOX and SSAT are activated by the inflammatory cytokine tumor necrosis factor (TNF)-α; SMOX can be similarly induced by interleukin-6 (Babbar and Casero 2006; Babbar et al. 2006b, 2007). Furthermore, oxidative stress itself, in the form of $H_2O_2$, has been shown to induce *SAT1* transcription and alter intracellular polyamine concentrations (Chopra and Wallace 1998; Smirnova et al. 2012). Additionally, the alcohol metabolite acetaldehyde was recently shown to induce spermine oxidation (Uemura et al. 2013), ischemia–reperfusion injury induces both SSAT and SMOX catabolic pathways (Zahedi et al. 2009; Zahedi and Soleimani 2011), and exposure to carbon tetrachloride induces SSAT in hepatocytes (Zahedi et al. 2012). Each of these stimuli and their disease implications are discussed in the following sections.

## 5.4 Physiological Consequences of Increased Polyamine Catabolism

The higher polyamines, spermine in particular, play important physiological roles in protection from oxidative stress. Enhanced polyamine catabolism reduces this protection while concomitantly generating ROS and toxic aldehyde by-products. As a result, increased polyamine catabolism, resulting from the stimuli just mentioned, has been implicated in several pathophysiological conditions, including neurological and liver disease, stroke, kidney failure, and cancer.

### *5.4.1 ROS Generation and Oxidative DNA Damage*

#### 5.4.1.1 Inflammation-Associated Hyperproliferative Conditions

Approximately 20 % of all human cancers can be causally linked to chronic inflammation, particularly through infection with human pathogens (Zur Hausen 2009). Representatives of these pathogens are also inducers of SMOX, and elevated levels of SMOX have been observed in several inflammation-associated human conditions that are risk factors for the development of epithelial cancers. These observations suggest a role for spermine oxidation in the initiation of tumorigenesis. In addition to generating oxidative stress capable of DNA damage in the epithelial cell, several systems have now also demonstrated inductions of the polyamine oxidases in infiltrating inflammatory cells as a potential means for immune response evasion.

An active area of investigation regarding infection and inflammation focuses on *Helicobacter pylori*, a gram-negative bacterium that infects the stomach mucosa and causes inflammation in the form of chronic gastritis and peptic ulcers (Hardbower et al. 2013). Although eliciting acute and chronic immune and inflammatory responses, *H. pylori* evades the antimicrobial mechanisms of the immune response and often persists for the life of the host (Gobert et al. 2001; Bussiere et al. 2005). Approximately 50 % of the world's population is infected with *H. pylori*, which is considered a class I carcinogen and is believed to be the causal agent of 95 % of gastric cancers (Malfertheiner et al. 2005).

Gastric epithelial cells respond to infection with *H. pylori* through an induction of SMOX mRNA and activity. The generation of $H_2O_2$ that results from SMOX induction by *H. pylori* has been causally linked to DNA damage and apoptosis in the gastric mucosae of humans and mice (Xu et al. 2004). Importantly, a subpopulation of gastric epithelial cells in which *H. pylori* infection has induced SMOX activity and high amounts of DNA damage remains resistant to apoptosis, therefore increasing the likelihood that these cells will undergo malignant transformation (Chaturvedi et al. 2011). SMOX-mediated $H_2O_2$ production in response to *H. pylori* is also induced in the host macrophages responding to the infection, resulting in

macrophage apoptosis and contributing to immune evasion and bacterial persistence (Chaturvedi et al. 2004, 2013). Recent studies with affected patient samples have further implicated the critical role played by SMOX in the etiology of *H. pylori*-induced gastric cancer (Chaturvedi et al. 2014b, 2014c).

Chronic inflammation and the associated oxidative damage is also a risk factor for the development of colorectal neoplasia. Induction of SMOX has been demonstrated in a mouse model of infection with the enterotoxigenic bacterium *B. fragilis* (ETBF), which results in ulcerative colitis, acute diarrheal disease, inflammatory bowel disease, and ultimately causes colon cancer. This SMOX induction has been implicated as the source of ROS-induced DNA damage in ETBF-infected colonic epithelium, and treatment with the SMOX inhibitor MDL72, 527 decreased ETBF-induced DNA damage, colonic inflammation, proliferation, and tumorigenesis (Goodwin et al. 2011). In patient samples of ulcerative colitis, SMOX protein expression in infiltrating mononuclear cells was found to correlate with the severity of inflammatory disease scoring, consistent with a role for SMOX in colitis pathogenesis (Hong et al. 2010).

Chronic inflammation in the form of prostatitis is believed to contribute to the development of prostate cancer. In prostate tissue samples from patients with prostate disease spanning the spectrum from inflammation to prostate adenocarcinoma, SMOX protein expression was increased in comparison with individuals without disease (Goodwin et al. 2008). When examining patient-matched samples, SMOX expression was increased in prostatic intraepithelial neoplasia (PIN) and prostate cancer samples relative to benign prostatic epithelium from the same individual, with the greatest increase observed in PIN lesions, which are recognized as precursors to prostate carcinoma. Consistent with the inflammation-associated conditions already described, these data suggest a role for SMOX in precursor lesion development and carcinogenic initiation events. Furthermore, patients who developed prostate disease demonstrated significantly higher SMOX expression even in the nondiseased areas of prostatic epithelium when compared to those without disease, suggesting increased SMOX expression is an important component early in the disease process.

*Pneumocystis* infection is also associated with upregulated polyamine catabolism. *Pneumocystis* opportunistically infects the lungs of immunocompromised patients and results in a decrease in alveolar macrophages. This apoptosis appears to be the result of $H_2O_2$ production by the macrophages through increased APAO activity subsequent to the induction of polyamine biosynthesis (Liao et al. 2009).

#### 5.4.1.2 Ischemia–Reperfusion and Toxin-Induced Injury

Renal ischemia–reperfusion has been shown to induce polyamine catabolism through SSAT and SMOX in both the kidney and liver, resulting in oxidative stress and apoptosis that do not occur in SSAT-deficient animals (Zahedi et al. 2009; Zahedi and Soleimani 2011). Furthermore, endotoxin, or LPS, is a major cause of sepsis-related acute kidney injury, and injection of mice with LPS resulted in inductions of renal SSAT and SMOX. Pharmacological inhibition of polyamine oxidation or ablation of SSAT decreased renal cell damage, implicating the catalysis of

polyamines in the mediation of endotoxin-induced acute kidney injury (Zahedi et al. 2010). In similar studies, it was demonstrated that exposing mice to carbon tetrachloride resulted in a large induction of hepatocyte SSAT activity that was associated with liver damage, and this damage was prevented by pharmacological inhibition of polyamine oxidation or SSAT ablation (Zahedi et al. 2012).

#### 5.4.1.3 HIV-Related Dementia

Chronic oxidative stress occurs in brain tissues of HIV-infected patients and is associated with the development of human immunodeficiency virus (HIV)-associated dementia. Recently, the HIV-1 *Tat* gene was shown to induce SMOX activity, resulting in ROS generation and a reduction in intracellular spermine content in neuroblastoma cells. These studies provided evidence that SMOX-derived $H_2O_2$ induces oxidative stress that plays a role in neuronal cell death and the etiology of dementia associated with HIV infection (Capone et al. 2013).

### *5.4.2 Acrolein Generation*

In addition to generating ROS, polyamine oxidation results in toxic aldehydes that have been implicated in neurological conditions and diseases (Pegg 2013). The aldehyde product of SMOX activity, 3-amidopropanal, can be spontaneously metabolized to acrolein, a major toxicity factor that is being actively investigated as a factor in several pathologies.

In this regard, multiple studies have implicated a role for polyamine oxidation-associated acrolein production in neuronal damage. APAO and SMOX proteins and acrolein adducts can be measured in the plasma of patients and hold potential as biomarkers for the detection of stroke (Igarashi and Kashiwagi 2011a, b; Tomitori et al. 2005). Acrolein conjugates, when combined with amyloid-β ratios, also provide an accurate indication for Alzheimer's disease, even in patients with only mild cognitive impairment (Waragai et al. 2012).

Increases in free and protein-conjugated acrolein have also been detected in various renal pathologies, including renal failure, and the metabolism of alcohol in the liver has been shown to induce spermine oxidation resulting in increased detection of acrolein (Sakata et al. 2003a, b; Uemura et al. 2013). This increase in hepatocellular SMOX suggests a mechanism for the liver toxicity and decreased regenerative abilities associated with chronic alcohol intake.

### *5.4.3 Reduction of Intracellular Polyamine Pools*

The studies described here focus on the cellular effects of toxic by-products generated through elevated polyamine catabolism; however, the resulting reduction in intracellular polyamine concentrations must also be considered, as their depletion

can significantly exacerbate the toxic outcome. Spermine and spermidine function to protect DNA from oxidative damage, and spermine has been shown to act directly as a free-radical scavenger (Ha et al. 1998a, b; Khan et al. 1992a, b; Rider et al. 2007; Nilsson et al. 2000). Therefore, elevated polyamine catabolism, particularly through nuclear SMOX induction, not only generates ROS but also functionally reduces the antioxidant levels of the cell.

Similarly, polyamines have been recognized as mediators of immune function by negatively regulating the production of certain inflammatory cytokines, including TNF-α and interleukin (IL)-1β, and nitric oxide (Zhang et al. 2000; Perez-Cano et al. 2003; Paul and Kang 2013) Therefore, the transcriptional activation of SSAT and SMOX by pro-inflammatory cytokines, such as TNF-α, decreases the abundance of spermine and spermidine that would normally repress further production of TNF-α, thus having the potential to exacerbate the chronic inflammatory response.

## 5.5 Polyamine Catabolism as a Therapeutic Target

### *5.5.1 Chemotherapeutic Strategies*

In the cancer setting, much research has focused on inducing polyamine catabolism in established tumors with the goal of reducing the natural polyamines required for tumor proliferation while selectively inducing tumor cell apoptosis through the generation of ROS (Nowotarski et al. 2013; Battaglia et al. 2013). Several classes of polyamine analogues have now been characterized to accomplish this goal in vitro; however, their clinical utility as single agents has been limited. Combining these analogues with other agents targeting the tumor cell has become a promising chemotherapeutic option. For example, cotreatment of small cell lung tumor cells with a bis(ethyl) polyamine analogue and the HDAC inhibitor MS-275 produced a synergistic induction of SSAT activity that was associated with growth inhibition (Murray-Stewart et al. 2013). Furthermore, these analogues have been observed to work in concert with other common cytotoxic agents, including 5-fluorouracil and platinum-based compounds, to induce polyamine catabolism (Hector et al. 2004, 2008; Pledgie-Tracy et al. 2010).

### *5.5.2 Chemopreventive Strategies*

SSAT induction is observed in response to treatment with NSAIDs, and this induction contributes to the antiproliferative activity of NSAIDs in regard to the development of colorectal carcinoma (Babbar et al. 2003, 2006a). Recently, a phase III clinical trial examined the chemopreventive potential of combining treatment with a specific NSAID (sulindac) with an inhibitor of polyamine biosynthesis (difluoromethylornithine) (Meyskens et al. 2008). Patients in this study who received the combination therapy demonstrated dramatic reductions in the occurrence of colon

polyps and adenomas, verifying the utility of targeting polyamine metabolism in conjunction with inflammation.

In light of the growing evidence supporting a role for polyamine oxidation in the etiologies of several human conditions and diseases, including cancer initiation, attention must also be given to attenuating this response as a means for chemoprevention. As SMOX activity has been demonstrated in the cell nucleus, its inhibition in the presence of cancer-predisposing, SMOX-inducing factors such as *H. pylori* infection appears to provide protection from the oxidative DNA damage that leads to carcinogenesis. It is likely that this inhibition of polyamine catabolism prevents the generation of ROS while maintaining the intracellular spermine pools capable of free-radical scavenging. Although effective inhibitors for SMOX exist, including MDL72527, they are nonspecific and also inhibit APAO. The identification of specific inhibitors of the polyamine oxidases will provide both experimental and therapeutic benefit.

## 5.6 Summary

In conclusion, although consisting of only three enzymes, intracellular mammalian polyamine catabolism is a dynamic process that is capable of responding to a multitude of environmental stimuli. Depending on the context, these interactions can have positive or negative cellular effects: polyamine catabolism can protect cells from the toxic effects of excessive polyamine accumulation and limit uncontrolled proliferation in instances of upregulated polyamine biosynthesis. Yet, excessive polyamine catabolism can cause oxidative stress and toxin generation with the potential to increase carcinogenic events, limit the regeneration or viability of essential cells, or diminish the innate immune response to infection. Thus, both targeted increases in polyamine catabolism and targeted inhibition of polyamine catabolism have the potential for therapeutic benefit, depending on the precise context of the changes occurring. Additional studies are necessary to fully understand and exploit these dynamic pathways for maximum therapeutic advantage.

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# Chapter 6
# Polyamine Catabolism in Plants

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**Abstract** The copper-dependent amine oxidases (CuAOs) and flavin-containing polyamine oxidases (PAOs) are involved in polyamine (PA) catabolic processes. Studies on plant CuAOs are still incomplete, whereas research on plant PAOs has advanced significantly in the past decade. The maize PAO, the best studied plant PAO, and the barley PAOs were shown to catalyze PAs in a terminal catabolic pathway. Therefore, plant PAOs were assumed to have terminal catabolic activity, which differs from the back-conversion activity of mammalian PAOs. However, plant PAOs that have back-conversion activity are now reported. Here, studies on PAOs from the two model species *Arabidopsis thaliana* and *Oryza sativa* are compiled, and research on CuAOs is updated. Our current understanding of the roles of PAOs and CuAOs in plant development and defense responses is described.

**Keywords** *Arabidopsis thaliana*, back-conversion pathway • Copper-dependent amine oxidase • *Oryza sativa*, plant, polyamine oxidase • Terminal catabolism pathway

## 6.1 Introduction

Polyamines (PAs) are involved in growth, development, and adaptation against various environmental changes in plants (Kusano et al. 2008; Alcázar et al. 2010; Bassard et al. 2010; Handa and Mattoo 2010; Mattoo et al. 2010; Takano et al. 2012). Major plant PAs are the diamines putrescine (Put) and cadaverine (Cad, abundant in legumes), the triamine spermidine (Spd), and the tetraamines spermine (Spm) and thermospermine (T-Spm). Cellular PA levels are regulated with a

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dynamic equilibrium between biosynthesis and catabolism. PA catabolism is catalyzed by two classes of amine oxidases: the copper-containing amine oxidases (CuAOs) and the polyamine oxidases (PAOs). CuAOs (EC 1.4.3.6) are dimers of identical 70- to 90-kDa subunits, and each subunit contains a single copper ion and a 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor (Medda et al. 1995b; Dawkes and Phillips 2001). Crystal structures have been solved for amine oxidases from *Escherichia coli* and pea seedlings (Møller and McPherson 1998). By contrast, PAOs (EC 1.5.3.11) are monomeric enzymes and contain a noncovalently bound flavin adenine dinucleotide (FAD) as a cofactor. The first characterized apoplastic maize PAO and barley PAOs oxidize PAs in a terminal catabolic pathway. The recently characterized *Arabidopsis* and rice PAOs oxidize PAs in an alternative pathway, the back-conversion pathway. The most updated information on plant CuAOs and PAOs is provided in this chapter. Specific reviews on plant PAOs are available (Cona et al. 2006; Angelini et al. 2010; Wimalasekera et al. 2011a; Moschou et al. 2012).

## 6.2 PAOs

### *6.2.1 Terminal Catabolic Pathway and Back-Conversion Pathway*

The PA catabolic pathway has been well studied in mammals. Spd/Spm $N^1$-acetyltransferase (SSAT; EC 2.3.1.57; Casero and Pegg 1993) modifies Spd and Spm before PAO action. This acetylation process is a rate-limiting step in the catabolic pathway (Wallace et al. 2003). A mammalian PAO oxidizes $N^1$-acetyl Spm and $N^1$-acetyl Spd at the carbon on the *exo*-side of the $N^4$-nitrogen to produce Spd and Put, respectively (Vujcic et al. 2002; Wu et al. 2003; Cona et al. 2006). Mammalian Spm oxidases (SMOs) and the yeast orthologue (encoded by *Fms1*) oxidize Spm at the carbon on the *exo*-side of the $N^4$-nitrogen to produce Spd, 3-aminopropanal, and $H_2O_2$ without acetyl modification (Wang et al. 2001; Vujcic et al. 2002; Cervelli et al. 2003); thus, mammalian PAOs and SMOs catalyze back-conversion reactions. The molecular evolution of the *PAO* and *SMO* genes has been discussed recently (Polticelli et al. 2012).

In plants, the first characterized maize and barley PAOs catalyze terminal catabolic reactions (Fig. 6.1) (Federico et al. 1990, 1996; Tavladoraki et al. 1998; Radova et al. 2001; Cervelli et al. 2001, 2004, 2006). This type of PAO oxidizes the carbon at the *endo* side of the $N^4$-nitrogen of Spm and Spd, producing *N*-(3-aminopropyl)-4-aminobutanal and 4-aminobutanal, respectively, as well as 1,3-diaminopropane and $H_2O_2$ in both reactions (Cona et al. 2006; Angelini et al. 2010). In 2006, Tavladoraki et al. reported that plants have a back-conversion type of PAO (Fig. 6.1). They showed that *Arabidopsis* AtPAO1 produces Spd from Spm and norspermidine from norspermine (Norspm). AtPAO1 is the first plant PAO known to catalyze a PA back-conversion reaction. The current consensus indicates that plants have two types of PAOs: one catalyzes a terminal catabolic reaction whereas the other catalyzes a PA back-conversion reaction.

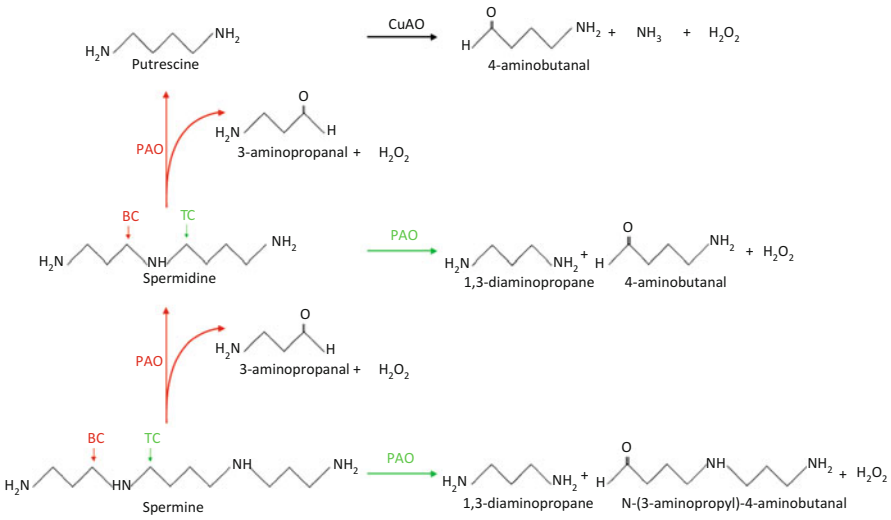


**Fig. 6.1** Schematic drawing of the polyamines (PA) catabolic pathways in plants. Diamine Put is converted to 4-aminobutanal along with ammonia and hydrogen peroxide ($H_2O_2$) by a reaction catalyzed by CuAO. On the other hand, triamine Spd and tetraamines, Spm and T-Spm, are catabolized by two alternative pathways: one is a terminal catabolism (*TC*) pathway (marked by *green arrows*) and the other is a back-conversion (*BC*) pathway (marked by *red arrows*). The positions of carbon oxidized by TC- and BC-type PAOs are also indicated with short *green* and *red arrows*, respectively

In the next sections, the characterization of PAOs in two model plants, *Arabidopsis thaliana* and *Oryza sativa*, is summarized.

### 6.2.2 *PAOs in* Arabidopsis thaliana

In *A. thaliana*, five *PAO* genes have been identified, which are named *AtPAO1* to *AtPAO5*. To date, four of the gene products, namely AtPAO1 to AtPAO4, have been biochemically characterized (Tavladoraki et al. 2006; Moschou et al. 2008, 2012; Kamada-Nobusada et al. 2008; Takahashi et al. 2010; Fincato et al. 2011, 2012). AtPAO1 localizes in the cytoplasm and oxidizes Spm, T-Spm, and Norspm, but not Spd, in a back-conversion reaction (Tavladoraki et al. 2006). AtPAO2, AtPAO3, and AtPAO4 localize in peroxisomes (Moschou et al. 2008; Kamada-Nobusada et al. 2008). All the peroxisomal PAOs show PA back-conversion activity, although they differ in PA specificity (Moschou et al. 2008; Kamada-Nobusada et al. 2008; Takahashi et al. 2010; Fincato et al. 2011). AtPAO2 and AtPAO3 convert Spm to Put via Spd, whereas AtPAO4 produces less Put from Spm, which is explained with the very low $k_{cat}$ value for Spd (Fincato et al. 2011). AtPAO5 localizes in the cytoplasm (Fincato et al. 2011; DWK unpublished data). In *Arabidopsis*, two PAOs are in the cytoplasm and the remaining three PAOs localize in the peroxisome, indicating that no PAOs exist in the apoplastic space (Table 6.1). AtPAO5 shows PA back-conversion

**Table 6.1** Characteristics of PAOs and CuAOs in *Arabidopsis thaliana* and *Oryza sativa*

| Gene name | Accession no. | Gene ID | Mode of reaction | Substrate Specificity | Subcellular localization | References |
|---|---|---|---|---|---|---|
| *A. thaliana* | | | | | | |
| *AtPAO1* | NM_121373 | At5g13700 | BC | T-Spm, Spm | Cytoplasm | Tavladoraki et al. (2006), Takahashi et al. (2010), Fincato et al. (2011, 2012) |
| *AtPAO2* | AF364952 | At2g43020 | BC | Spd, Spm, T-Spm | Peroxisome | Kamada-Nobusada et al. (2008), Takahashi et al. (2010), Fincato et al. (2011, 2012) |
| *AtPAO3* | AY143905 | At3g59050 | BC | Spd, Spm, T-Spm | Peroxisome | Moschou et al. (2008), Takahashi et al. (2010), Fincato et al. (2011, 2012) |
| *AtPAO4* | AF364953 | At1g65840 | BC | Spm, T-Spm | Peroxisome | Kamada-Nobusada et al. (2008), Takahashi et al. (2010), Fincato et al. (2011) |
| *AtPAO5* | AK118203 | At4g29720 | BC | Spm, T-Spm | Cytoplasm | Fincato et al. (2012), Kim et al. (in preparation) |
| *O. sativa* | | | | | | |
| *OsPAO1* | NM_001050573 | Os01g0710200 | BC | Spm, T-Spm | Cytoplasm | Liu et al. (2014a) |
| *OsPAO2* | NM_001055782 | Os03g0193400 | n.d. | | n.d. | |
| *OsPAO3* | NM_001060458 | Os04g0623300 | BC | Spd, Spm, T-Spm | Peroxisome | Ono et al. (2012) |
| *OsPAO4* | NM_001060753 | Os04g0671200 | BC | Spm, T-Spm | Peroxisome | Ono et al. (2012) |
| *OsPAO5* | NM_001060754 | Os04g0671300 | BC | Spm, T-Spm | Peroxisome | Ono et al. (2012) |
| *OsPAO6* | NM_001069545 | Os09g0368200 | TC (?) | | n.d. | |
| *OsPAO7* | NM_001069546 | Os09g0368500 | TC | Spm, Spd | Apoplast | Liu et al. (2014b) |
| *A. thaliana* | | | | | | |
| *ATAO1* | NM_117580 | At4g14940 | TC | Put | Apoplast | Møller and McPherson (1998) |
| *AtCuAO1* | NM_104959 | At1g62810 | TC | Put, Spd | Apoplast | Wimalasekera et al. (2011b), Planas-Portell et al. (2013) |
| *AtCuAO2* | NM_102906 | At1g31710 | TC | Put, Spd | Peroxisome | Planas-Portell et al. (2013) |
| *AtCuAO3* | AY120717 | At2g42490 | TC | Put, Spd | Peroxisome | Planas-Portell et al. (2013) |

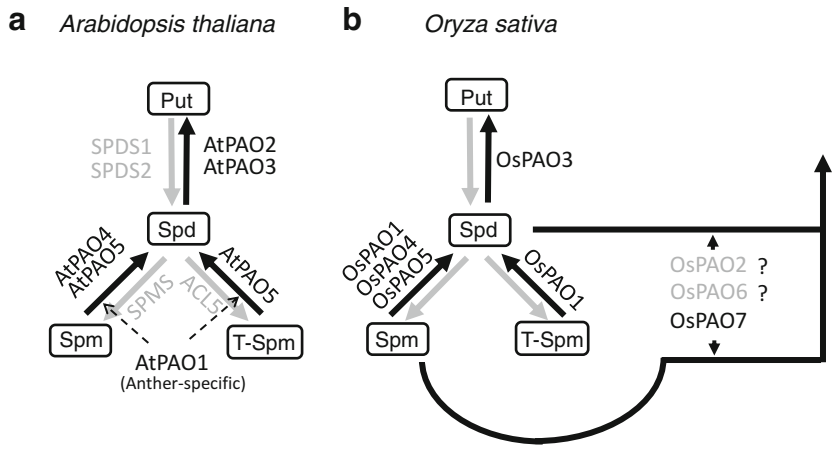


**Fig. 6.2** Main reactions catalyzed by PAOs in *Arabidopsis* and rice plants. **a** All five PAOs back-convert different PAs in *Arabidopsis thaliana*. **b** Four PAOs of seven back-convert different PAs, and two PAOs of the remaining three catabolize PAs by the terminal catabolic pathway

activity with a preference for Spm and T-Spm (DWK, unpublished data). Tavladoraki et al. (2006) reported that recombinant AtPAO1 produced very small amounts of 1,3-diaminopropane (DAP) during Spm oxidation, suggesting that this enzyme oxidizes Spm through a terminal catabolic pathway, although this activity is very low compared to that of its back-conversion activity. In summary, all *Arabidopsis* PAOs primarily catalyze back-conversion reactions (Table 6.1, Fig. 6.2a).

*Arabidopsis PAO*s exhibit distinct and organ-specific expression patterns. *AtPAO1* is expressed primarily in the root transition region between the apical meristem and the elongation zone, and it is also expressed in anthers. *AtPAO2* is expressed in the quiescent center, columella initials, and pollen, whereas *AtPAO3* is expressed in columella, guard cells, and pollen. *AtPAO4* is expressed in whole roots and all flower organs except petals, and *AtPAO5* is expressed in the vascular system of roots and hypocotyls (Takahashi et al. 2010; Fincato et al. 2012). Abscisic acid (ABA)-induced expression of *AtPAO1* in root tip and *AtPAO2* in guard cells has been reported (Fincato et al. 2012). These results suggest that each AtPAO has distinct physiological roles.

### *6.2.3 PAOs in* Oryza sativa

This monocotyledonous model plant harbors seven *PAO* genes, sequentially designated *OsPAO1* to *OsPAO7*. Three *PAO*s, namely, *OsPAO3*, *OsPAO4*, and *OsPAO5*, are expressed at higher levels both at the seedling stage and at the reproductive stage compared to those of the other four *OsPAO*s. The gene products contain peroxisomal-targeting signals in their carboxy-termini, and they localize in peroxisomes

(Table 6.1, Fig. 6.2b) (Ono et al. 2012). OsPAO3, OsPAO4, and OsPAO5 have PA back-conversion activity (Ono et al. 2012). Studies on *Arabidopsis* and rice PAOs suggest that, even in other plants, peroxisome-localized PAOs could be predicted to have back-conversion activity. *OsPAO1* lacks introns, similar to that of the *Arabidopsis AtPAO5*. *OsPAO1* expression is induced by treatment with tetraamine, Spm, or T-Spm. In *OsPAO1*-promoter *GFP*-transgenic rice plants, the initially observed GFP signal in the root transition region between the apical meristem and the elongation zone is expanded in the elongation zone by tetraamine treatment (Liu et al. 2014a). Consistent with the specific induction by tetraamines, recombinant OsPAO1 prefers tetraamines as substrate, and back-converts them to Spd, but not further to Put. This enzyme shows different pH optima, with pH 6.0 for T-Spm and pH 8.5 for Spm (Liu et al. 2014a). OsPAO1 localizes in the cytoplasm. The remaining three OsPAOs, namely, OsPAO2, OsPAO6, and OsPAO7, resemble each other, and show high identity to the maize ZmPAO and the barley HvPAO1 and HvPAO2, suggesting that they function in a terminal catabolic pathway. The recombinant OsPAO7 produces DAP from Spm and Spd, demonstrating that OsPAO7 is a terminal catabolic-type enzyme (Liu et al. 2014b). OsPAO7 is located in the peripheral boundary of the plant cell, possibly through its amino-terminal signal peptide and transmembrane sequence. *OsPAO7* is expressed in flower organs, especially anther walls and pollen, but not in pistils (Liu et al. 2014b). The peroxisomal *OsPAO* members *OsPAO3*, *OsPAO4*, and *OsPAO5* are also expressed in anthers (Liu et al. 2014b).

In *Oryza sativa*, four OsPAOs, namely, OsPAO1, OsPAO3, OsPAO4, and OsPAO5, function in a back-conversion pathway, whereas two OsPAOs, OsPAO7 and possibly OsPAO6, function in a terminal catabolic pathway. Because of the rather long truncation at its amino-terminal region, OsPAO2 may not be a functional enzyme. Even in *O. sativa*, the PA back-conversion pathway is a dominant route of PA catabolism. The terminal catabolic pathway may function in specific tissues and/or during specific developmental stages (Table 6.1, Fig. 6.2b) (Liu et al. 2014b). To explore the distinct roles of OsPAOs in various physiological processes, genetic approaches such as gene silencing by RNAi are required.

## 6.3 CuAOs

### *6.3.1 General Information on Plant CuAOs*

Molecular features, substrate specificities, inhibitors, stoichiometry of the catalyzed reaction, spectroscopic features, prosthetic groups, and reaction mechanisms of plant CuAOs have been reviewed previously (Medda et al. 1995a). CuAOs are homodimers in which each subunit, consisting of approximately 670–780 amino acids, contains a copper ion and a redox cofactor TPQ, generated by posttranslational autocatalytic modification from an active-site tyrosine residue (Møller and McPherson 1998; Dawkes and Phillips 2001). CuAOs have rather diverse sequences, and only approximately 30 amino acid residues are fully conserved (Tipping and

McPherson 1995; Møller and McPherson 1998; Planas-Portell et al. 2013). Of those residues, 3 conserved histidine residues may be the ligands for copper binding, and tyrosine at the 406 position is modified to TPQ in pea CuAO. Plant CuAOs generally catalyze the oxidation of the diamines Put and Cad at the primary amino group to give 4-aminobutylaldehyde and 5-aminopentylaldehyde, which spontaneously cyclize to $\Delta^1$-pyrroline and $\Delta^1$-piperideine, respectively, along with ammonia and $H_2O_2$ (Federico and Angelini 1991; Medda et al. 1995a).

### 6.3.2 *CuAOs in* Arabidopsis thaliana

Although *Arabidopsis* contains at least ten CuAO-like genes, only four genes have been biochemically characterized, including *AtAO1* (At4g14940), *AtCuAO1* (At1g62810), *AtCuAO2* (At1g31710), and *AtCuAO3* (At2g42490) (Table 6.1) (Møller and McPherson 1998; Planas-Portell et al. 2013). AtAO1 produced in insect Sf9 cells oxidizes Put but not Spd. *AtAO1* expression is observed in vascular tissue and root-cap cells, both of which are destined to undergo programmed cell death (Møller and McPherson 1998). AtCuAO1, AtCuAO2, and AtCuAO3 oxidize Put and Spd but not Spm (Planas-Portell et al. 2013). AtCuAO1 is an extracellular protein such as AtAO1, whereas AtCuAO2 and AtCuAO3 are localized in peroxisomes. Temporal expression and hormonal responses of *AtCuAO1* to *AtCuAO3* have been reported. *AtCuAO1* is responsive to ABA and salicylic acid (SA), *AtCuAO2* is responsive to methyl jasmonate (MeJA) and wounding, and *AtCuAO3* responds to several stimuli such as ABA, SA, flagellin, and MeJA, but not to wounding or the ethylene precursor ACC (Planas-Portell et al. 2013). Tun et al. (2006) showed that exogenously applied PAs rapidly produced nitric oxide (NO) in *Arabidopsis*. The group further demonstrated that *Arabidopsis AtCuAO1* was involved in PA-induced NO production (Wimalasekera et al. 2011b).

## 6.4 CuAO and PAO Are Involved in Crucial Biological Processes

### 6.4.1 *Root Development and Xylem Differentiation*

Treatment with a specific PAO inhibitor attenuates both Spd-induced root cell growth inhibition and Spd-induced cell-cycle arrest. The PAO inhibitor also disrupts differentiation of the secondary wall of meta-xylem elements and xylem parenchymal cells. Overexpression of maize *PAO* in tobacco plants induces programmed cell death (PCD) in root-cap cells (Tisi et al. 2011). The results suggest that $H_2O_2$ produced by Spd oxidation triggers secondary wall deposition and induces PCD. A link between PAs and PCD has been reviewed recently by Moschou and Roubelakis-Angelakis (2014).

### 6.4.2 Pollen Tube Growth

Pollen contains high levels of PAs, which may be explained by high activities of PA biosynthetic enzymes (Song et al. 2002). PAs are proposed to play a role in pollen tube growth (Bagni et al. 1981; Song et al. 2002; Antognoni and Bagni 2008). Recently, a link between pollen tube growth and PAO was reported. Spd oxidase-derived $H_2O_2$ triggered the opening of hyperpolarization-activated $Ca^{2+}$-permeable channels in the pollen plasma membrane and enhanced pollen tube growth (Wu et al. 2010). Those authors showed that two allelic *Atpao3* loss-of-function mutants exhibited reduced pollen tube growth and seed number (Wu et al. 2010).

### 6.4.3 Salinity Stress

Su et al. (2007) show that γ-aminobutyric acid (GABA) produced by CuAO-mediated PA degradation plays a critical role in salinity stress. The involvement of AtCuAO1 in ABA-induced NO production suggests that CuAO is involved in the intermediate signaling pathway of ABA-mediated environmental stress responses, in which *rd29A* and *ADH1* expression is induced (Wimalasekera et al. 2011b). The involvement of PAO in abiotic and biotic adaptation is suggested. Under high salinity, reactive oxygen species produced by PAO activity in the apoplast sustain maize leaf growth (Rodriguez et al. 2009).

### 6.4.4 Pathogen Response

Walters (2003) indicated that CuAO activity was higher in incompatible interactions between plants and pathogens. For example, CuAO activity is high during the interaction of barley and powdery mildew fungus or that of chickpea and *Asochyta rabiei* (Angelini et al. 1993; Walters et al. 2002). PAO-derived $H_2O_2$ triggers the hypersensitive response (HR) in tobacco plants following infection with tobacco mosaic virus (TMV), and after treatment with cryptogein, a protein elicitor secreted by the oomycete *Phytophthora cryptogea* (Yoda et al. 2003, 2006). In tobacco plants carrying the *N* gene and the TMV system, the accumulation of apoplastic Spm is reported in response to TMV infection (Yamakawa et al. 1998). Takahashi et al. (2003, 2004) showed that this apoplastic Spm induces a subset of HR genes through mitochondrial dysfunction, and the resulting activation of two mitogen-activated protein kinases. In *Arabidopsis* and the cucumber mosaic virus (CMV) system, Spm-triggered defense gene induction was described. Treatment with the PAO inhibitor suppressed defense gene activation and compromised the defense response against CMV (Mitsuya et al. 2009). Marina et al. (2008) reported that tobacco plants infected by either the necrotrophic fungus *Sclerotinia sclerotiorum* or

by the biotrophic bacterium *Pseudomonas viridiflava* had higher PA levels and greater necrosis, which functioned to minimize growth of the nectrophic fungus, although it was beneficial for the biotrophic bacterium. Exogenously applied T-Spm and ectopic expression of *ACL5* (T-Spm synthase gene) increased *Arabidopsis* resistance to the biotrophic bacterium. The phenomenon was blocked by a PAO inhibitor, suggesting a role for T-Spm oxidation (Marina et al. 2013). Exogenously applied T-Spm restricted CMV multiplication via induced expression of a subset of pathogen-responsive *Arabidopsis* genes (Sagor et al. 2012). In all these cases, PAO is a crucial protein, and its reaction products ($H_2O_2$ and/or the aldehyde) play important roles in plant–pathogen interactions in the apoplastic space (Moschou et al. 2009).

## 6.5 Perspectives

The understanding of CuAOs and PAOs in *Arabidopsis* and PAOs in *Oryza sativa* has progressed significantly in recent years. Information on cellular localization, reaction chemistry, substrate specificity, and spatiotemporal expression patterns of CuAO and PAO family members is available. The involvement of CuAO and PAO in several physiological processes has been documented. However, most reports of physiological roles of CuAO and PAO are based on data using specific inhibitors. Knockout lines of specific CuAO or PAO genes and future research with advanced technologies will provide useful new data. New knowledge of the reaction mechanisms and physiological roles of CuAOs and PAOs can yield new strategies to produce higher-biomass plants and generate abiotic stress-tolerant plants by manipulating PA catabolic genes and PA levels.

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# Part III
# Polyamine Homeostasis

# Chapter 7
# Antizyme

**Noriyuki Murai**

**Abstract** Antizyme (AZ) first emerged as an inhibitor of ornithine decarboxylase (ODC), a key enzyme in the polyamine biosynthesis. Expression of AZ rises in response to increasing cellular polyamine levels through the polyamine-induced translational frameshifting mechanism. Synthesized AZ proteins bind to an ODC monomer and trigger its degradation by the 26S proteasome in a ubiquitin-independent manner. To reduce the cellular polyamine level, AZ also inhibits the uptake of extracellular polyamine. Therefore, AZ provides the feedback regulation of cellular polyamines. In mammals, cells express three members of the AZ protein family: AZ1-3, AZ1, and AZ2, are distributed in most tissues whereas AZ3 is testis specific. AZ is regulated by protein antizyme inhibitors (AZINs) that are homologous to ODC but lack the enzymatic activity. Two isoforms of AZINs, AZIN1 and AZIN2, are known. This chapter reviews the function and regulation of AZs and AZINs.

**Keywords** Antizyme • Antizyme inhibitor • Ornithine decarboxylase • Proteasome • Translational frameshifting • Ubiquitin-independent degradation

## 7.1 Antizyme 1

Antizyme 1 (AZ1) is the most widely studied among the antizyme (AZ) family (Table 7.1). AZ1 was initially described as a polyamine-induced inhibitor of ornithine decarboxylase (ODC) in mammalian cells (Heller et al. 1976). AZ is widely distributed among eukaryotes from yeast to humans (Ivanov et al. 2000a). AZ1 binds to the ODC monomer, inhibits enzyme activity, and targets it to the 26S proteasome for proteolytic degradation without ubiquitination (Murakami et al. 1992; Li and Coffino 1994; for review, see Hayashi et al. 1996; Coffino 2001). AZ1 itself is also degraded by the proteasome but in a ubiquitin-dependent manner (Gandre et al. 2002). The half-life of ODC is very short (about 15 min) in the presence of AZ1 (Murakami et al. 1992). AZ1-dependent acceleration of ODC degradation requires two structurally important regions on ODC: one is the AZ-binding element

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**Table 7.1** Antizyme family

| | AZ1 | AZ2 | AZ3 |
|---|---|---|---|
| Species distribution | Yeast-mammals | Vertebrates | Mammals |
| Evolutional conservation | Lower | Higher | Lower |
| Tissue distribution | Whole body | Whole body | Testis |
| Expression level | High | Low | Low |
| Translational frameshifting | + | + | + |
| Induction by polyamines | + | + | + |
| ODC binding/inhibition | + | + | + |
| ODC degradation | + | + | − |
| In vitro | + | − | − |
| In vivo | + | + | − |
| AZ inhibitor binding | + | + | + |
| Inhibition of polyamine uptake | + | + | + |

located between residues 117 and 140 (Li and Coffino 1992), and the other is the C-terminal short region, especially five residues at the C-terminus (Hoyt et al. 2003). The C-terminal region is structurally flexible and its conformation is possibly altered by binding of AZ1 to ODC (Murakami et al. 1999; Almrud et al. 2000). The 26S proteasome may recognize the C-terminal region that is exposed by AZ1 binding to feed the ODC polypeptide in the proteasome cavity (Coffino 2001). To reduce the cellular polyamine level, AZ1 also inhibits the uptake of extracellular polyamine (Mitchell et al. 1994; Suzuki et al. 1994). The molecular target of AZ1 for this inhibition is not known because the main polyamine transporter has not been identified in mammals.

It had been believed that ODC is the only protein that AZ binds and accelerates its degradation. However, there are several reports that AZ1 binds to proteins other than ODC and accelerates their degradation (Table 7.2). The cell-cycle regulatory protein cyclin D1 is one of those proteins. Newman et al. (2004) demonstrated that polyamine induction and overexpression of AZ1 accelerate the degradation of cyclin D1 and siRNA for AZ1 stabilizes it. Both Aurora A and Mps1 are protein kinases related to cell division (Bischoff and Plowman 1999; Fisk and Winey 2001). Aurora A is important for inducing centrosome amplification (Bischoff et al. 1998; Zhou et al. 1998). Mps1 has the key roles in regulating the spindle assembly checkpoint and chromosome microtubule attachments. Although these proteins were known to be ubiquitinated and degraded by the proteasome (Cui et al. 2010; Littlepage and Ruderman 2002; Taguchi et al. 2002), it was reported that AZ1-mediated degradation pathways also exist (Lim and Gopalan 2007; Kasbek et al. 2010). Delta-N (DN) p73 is the amino-terminal truncated form of p73, a homologue of p53, lacking the transactivation domain. DNp73 acts as a dominant-negative inhibitor of both p73 and p53 and exhibits antiapoptotic properties. Dulloo et al. (2010) reported that AZ1 promotes DNp73 degradation ubiquitin independently but c-Jun dependently. However, these findings are still controversial. Bercovich et al. (2011) showed that AZ1 (and also AZ2)-mediated stimulation of degradations of

**Table 7.2** AZ1 contributes to degradation of several proteins other than ornithine decarboxylase (ODC)

| Proteins | Function | Characteristics of degradation | Degradation | References |
|---|---|---|---|---|
| Smad1 | BMP signaling pathway | Formation of HsN3 (β-subunit for 20S proteasome)-Smad1-AZ1 ternary complex | In vivo | Lin et al. (2002) |
| Cyclin D1 | Cell-cycle regulation | Direct interaction with AZ1 | In vitro<br>In vivo | Newman et al. (2004) |
| Aurora-A | Kinase<br>Cell division (important for progression of $G_2$/M phase) | Formation of AUKAIP1-AZ1-Aurora-A ternary complex | In vivo | Lim and Gopalan (2007) |
| DeltaNp73 | Regulation of apoptosis | Degradation is induced by DNA damage and c-jun dependent | In vivo | Dulloo et al. (2010) |
| Mps1 | $G_1$/M phase kinase<br>Cell division (amplification of centrosome) | Expression level of AZ1 affects the level of Mps1 at the centrosome | In vivo | Kasbek et al. (2010) |

*BMP* bone morphogenetic protein

cyclin D1, Aurora-A, and DNp73 are negligible and that AZ1 and 2 affect cell growth and viability solely through cellular polyamine metabolism . Further investigations are needed to solve the arguments whether AZs have targeting proteins for degradation other than ODC in the cells.

The mechanism for expression of AZ protein, which is called "polyamine-induced translational frameshifting," is unique. AZ1 mRNA consists of two open reading frames, ORF1 and ORF2. Translation of AZ mRNA is initiated at a start codon for ORF1, and only ORF1 product is produced by normal translation. With increased polyamine concentration, the reading frame changes to +1 direction at the end of ORF1 and ORF2 product is synthesized as a fusion of ORF1 product. This full-length AZ1 downregulates ODC and inhibits polyamine uptake (see following), and thus cellular polyamine concentration is regulated in a negative feedback manner (Matsufuji et al. 1995; Ivanov et al. 2000a) (Fig. 7.1). Three cis-acting elements on AZ1 mRNA are known to be necessary for the frameshifting (Fig. 7.2). The first is a 50-nucleotide (nt) sequence just 5′ of the frameshift site, which stimulates the frameshifting 2.5- to 5 fold (Howard et al. 2001). The second is the UGA stop codon of ORF1, which stimulates the frameshifting 15 to 20 fold. The third is a pseudoknot structure of mRNA that consists of about 60 nt downstream to the stop codon of ORF1 and gives 2.5- to 5-fold frameshift stimulation (Matsufuji et al. 1995; Petros et al. 2005). It remains unclear how polyamines stimulate the frameshifting in mammalian cells. It was reported in yeast that nascent AZ polypeptide was a relevant polyamine sensor (Kurian et al. 2011). Their model is that increased polyamines bind to the nascent AZ polypeptide to release the ribosome stalling on AZ mRNA.

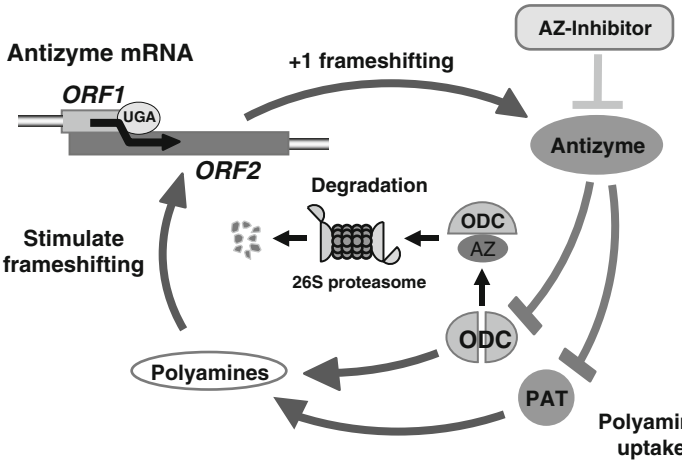


**Fig. 7.1** Schematic representation of feedback regulation of cellular polyamines mediated by antizyme (*AZ*) and antizyme inhibitor (AZIN). AZ mRNA consists of two open reading frames, *ORF1* and *ORF2*. Translation of AZ mRNA is initiated at a start codon for ORF1 and only ORF1 product is produced by normal translation. With an increased polyamine concentration, reading frame changes to +1 direction at the end of ORF1 and ORF2 product is synthesized as a fusion of ORF1 product. This full-length AZ1 binds to ODC monomer and targets it to the 26S proteasome for ubiquitin-independent degradation. Full-length AZ1 also inhibits polyamine uptake. Thus, cellular polyamine concentration is regulated in a negative feedback manner. *PAT* polyamine transporter

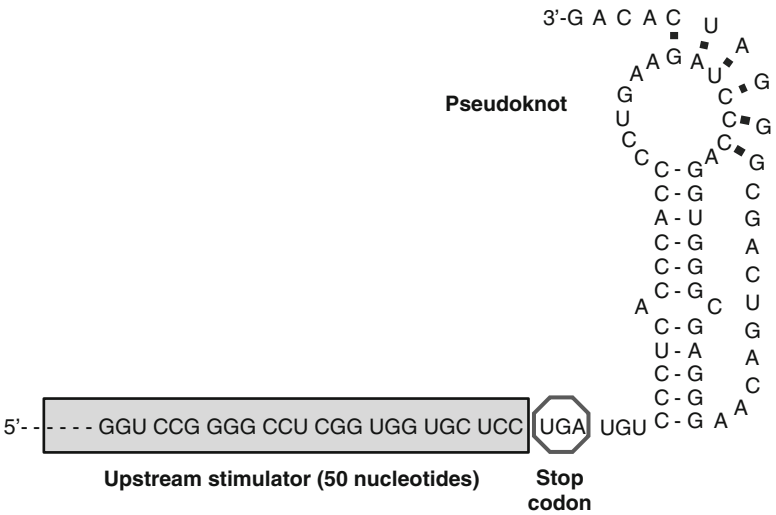


**Fig. 7.2** cis-acting elements for polyamine-induced antizyme frameshifting. Three cis-acting elements on AZ1 mRNA are known to be necessary for the frameshifting. The first is a upstream stimulator (50 nucleotide sequence just 5′ of the frameshift site). The second is a UGA stop codon of ORF1. The third is a pseudoknot structure of mRNA that consists of about 60 nt downstream to the stop codon of ORF1. These cis-acting elements stimulate the frameshifting 2.5–20 fold (see text)

## 7.2 Antizyme 2

Antizyme 2 (AZ2) is distributed widely in vertebrate tissue, as is AZ1, but the expression level is lower (Ivanov et al. 1998). AZ2 is one of the genes upregulated by a seizure-inducing drug in neuronal cells (Kajiwara et al. 1996) and later identified as a member of the AZ family (Ivanov et al. 1998). AZ2 has a very similar function to AZ1; AZ2 binds to the ODC monomer and targets it to the 26S proteasome for degradation (Chen et al. 2002) and inhibits polyamine uptake in the cells, but it does not promote ODC degradation in vitro (Chen et al. 2002; Murai et al. 2009). Interestingly, however, AZ2 is evolutionally more conserved across species than AZ1 (Ivanov et al. 2000a). We have recently found that AZ2, but not AZ1, is phosphorylated at Ser-186 and also showed that subcellular localization of AZ2 is mainly in the nucleus (Murai et al. 2009). AZ2 may have unique roles in the nucleus. The difference between AZ1 and AZ2 on the in vitro degradation of ODC might be caused by the difference of subcellular localizations. AZ2 is implicated in a type of human pediatric cancer, neuroblastoma. Low AZ2 mRNA expression correlates with unfavorable patient survival, whereas expression levels of other AZs and AZINs showed no correlation (Geerts et al. 2010). These results imply a tumor-specific role as well as neuron-specific roles of AZ2.

## 7.3 Antizyme 3

Antizyme 3 (AZ3/OAZ-t) was first identified as a haploid male germ cell-specific protein in mice (Tosaka et al. 2000; Ivanov et al. 2000b). Expression of AZ3 mRNA and protein were restricted to the testis (Ivanov et al. 2000b; Ike et al. 2002). The translation product of AZ3 mRNA is approximately 22 kDa and considered to be produced by a translational frameshifting mechanism as are AZ1 and AZ2. AZ3 binds to ODC but inhibition of ODC is weak (Ivanov et al. 2000b). AZ3 does not stimulate ODC degradation by the proteasome (Snapir et al. 2009). Tokuhiro et al. (2009) generated AZ3 knockout (AZ3-KO) mice and found that male homozygous AZ3-KO mice were infertile whereas females were unaffected. Although polyamine concentrations of epididymides and testes were normal, and sperm was successfully recovered from epididymides of the AZ3-KO mice, connection between head and tail of the sperm cells was fragile and easily separated. It has been reported that AZ3 interacts with testicular germ cell-specific protein GGN1, which was expressed from late pachytene spermatocytes to round spermatids (Zhang et al. 2005), but its physiological significance remains unknown. In contrast to these studies, Ruan et al. (2011) reported that the main product of AZ3 mRNA is approximately 12 kDa, which is encoded by ORF1 with an alternative CUG initiation codon located on the 5′-untranslated region, and that a putative 22-kDa frameshifted protein was not detected in rat testis. The 12-kDa protein modulates activity of protein phosphatase 1 through binding to myosin phosphatase targeting subunit 3 (MYPT3), which is present in spermatids and sperms. Further studies are needed to clarify the expression and function of AZ3 in testis.

## 7.4 Antizyme Inhibitor 1 (AZIN1)

AZIN1 was found as a protein that inhibits AZ activities (Fujita et al. 1982). AZIN1 binds to AZs with higher affinity than does ODC, and consequently leads to release of ODC from the AZ-ODC complex (Kitani and Fujisawa 1989). Thus, AZIN1 functions as a positive regulator of cellular polyamines. AZIN1 was cloned in 1996 and shown to be a homologue of ODC (Murakami et al. 1996). AZIN1 also resembles ODC in secondary structure, dimeric structure, molecular weight, and AZ-binding activity but lacks the catalytic activity. There are two isoforms of AZIN, AZIN1 and AZIN2, in mammalian cells. AZIN1 is ubiquitously distributed in most tissues and has a prominent role in cell proliferation and tumorigenesis (Olsen and Zetter 2011). Overexpression of AZIN1 in cultured cells provides a growth advantage to cells through neutralization of AZ functions (Keren-Paz et al. 2006). It was reported that both AZIN1 and AZ1 are localized at centrosomes, and that alterations in the AZ1/AZIN1 balance contribute to regulation of centriole amplification (Mangold et al. 2008). The change of AZIN1 level in centrosome causes the change of local AZ1, possibly leading to change in the level of Mps1, a potential target of AZ1 for proteasomal degradation (Kasbek et al. 2010). Murakami et al. (2009) showed that expression of AZIN1 increased at $G_1$ and $G_2$/M phases preceding the peak of ODC activity, and at that phase, rate of AZIN1 turnover become twofold that of interphase. In addition, subcellular localization of AZIN1 changes in response to the cell-cycle phases. Thus, AZIN1 might have a function to regulate local AZ and ODC levels during the cell cycle by changing its subcellular localization and expression level.

## 7.5 Antizyme Inhibitor 2 (AZIN2)

AZIN2 was first discovered in 2001 as an ODC paralogue lacking ODC activity (Pitkänen et al. 2001). AZIN2 interacts with all three AZs (AZ1–AZ3) and stimulates polyamine uptake as AZIN1. Distribution of AZIN2 is limited to certain tissues including the brain and testis, and in the testis the relative expression level is 25 fold higher than AZIN1 (López-Contreras et al. 2008). Hence, AZIN2 is suspected to function mainly in testes. Recent work showed that AZIN2 is also expressed in serotonin-containing granules of mast cells and that activation of mast cells rapidly upregulates AZIN2 (Kanerva et al. 2009). In addition, it was reported that AZIN2 localizes to post-Golgi secretary vesicles and regulates their transport by locally activating ODC and polyamine biosynthesis (López-Contreras et al. 2009; Kanerva et al. 2010). Thus AZIN2 plays distinct regulatory roles.

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# Chapter 8
# Bacterial Antizyme

**Yoshiyuki Kamio, Yoshihiro Yamaguchi, and Jun Kaneko**

**Abstract** A 22-kDa protein (P22), a direct counterpart to mammalian ornithine decarboxylase antizyme (ODC-AZ), was found in *Selenomonas ruminantium*, a gram-negative and anaerobic bacterium from sheep rumen. P22 does not degrade lysine/ornithine decarboxylase (LDC/ODC) but does bind to the AZ-binding region of LDC/ODC and triggers ATP-dependent proteolysis. This proteolytic system is totally compatible with the factors implicated in the AZ-mediated regulatory systems of mammalian ODC, such as mouse AZ or 26S proteasomes. Surprisingly, P22 is an L10 protein, a component of the large subunit of the bacterial ribosome. Interestingly, our research on bacterial antizyme was originally initiated from the study on the peptidoglycan of *S. ruminantium*, which contained covalently linked cadaverine, representing quite unusual characteristic among bacterial cell wall. In this chapter, we describe the chemical structure and biological function of the cadaverine-containing peptidoglycan of *S. ruminantium*, and the cellular biosynthesis of cadaverine by LDC/ODC and its P22 (=L10)-mediated regulation. In addition, we briefly refer to the phylogenetic distribution of LDC/ODC and ribosomal L10 among bacteria.

**Keywords** 22-kDa protein P22 • Bacterial antizyme • Cadaverine • Lysine/ornithine decarboxylase • Ribosomal protein L10 • *Selenomonas ruminantium*

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## 8.1 Finding of Polyamine in Peptidoglycan

### *8.1.1 Presence of Cadaverine Covalently Linked to the Peptidoglycan and Its Biological Function in* Selenomonas ruminantium

*Selenomonas ruminantium*, a gram-negative and anaerobic bacterium from sheep rumen, possesses a typical gram-negative cell surface, comprising a cytoplasmic membrane, peptidoglycan, and an outer membrane. However, it is characterized by the absence of murein-lipoprotein (MLP), which has an important role in the structural linkage between the outer membrane and peptidoglycan, thereby maintaining the structural integrity of the cell surface of gram-negative bacteria (Kamio and Takahashi 1980). The *Escherichia coli lpo* mutant lacks MLP and becomes hypersensitive to EDTA, resulting in rapid cell lysis upon exposure to it (Suzuki et al. 1978; Yem and Wu 1978). However, *S. ruminantium* cells were found to be resistant to EDTA despite the absence of MLP (Kamio and Takahashi 1980). This phenotypic difference led to the assumption that some entity might play a similar role as MLP, and further investigation of the peptidoglycan component was performed. The HCl-hydrolysate of a peptidoglycan preparation from a $^{14}$C-amino acid-labeled *S. ruminantium* cells was analyzed and it was found that there was a radioactive spot besides glucosamine, muramic acid, D-glutamic acid, *meso*-diaminopimelic acid, and DL-alanine. Furthermore, the radioactive spot is ninhydrin positive. The compound corresponding to this spot was isolated and analyzed chemically, and was identified as cadaverine dihydrochloride. Based on the chemical analysis of the peptidoglycan, it was determined that its primary peptide structure is L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine, and that the amino group of cadaverine covalently links to the α-carboxyl group of the D-glutamic acid residue through an amide bond (Kamio et al. 1981).

The biological function of the cadaverine covalently linked peptidoglycan was first clarified using DL-α-difluoromethyllysine (DFML), a potent and irreversible inhibitor of lysine decarboxylase (LDC) in vitro and in vivo (Kamio et al. 1986). In the presence of the inhibitor, *S. ruminantium* cells showed a significant decrease in the amount of cadaverine covalently linked to the peptidoglycan. They also exhibited growth inhibition accompanied by drastic morphological change and aberrant cell-surface structure, with detachment of the outer membrane from the peptidoglycan. The inhibitory effect was completely reversed by adding external free cadaverine, which was exclusively incorporated into the peptidoglycan. On the basis of these results, cadaverine is thought to play a significant role in maintaining the integrity of the cell surface.

The mechanism for the maintenance of cell-surface integrity, and the precise role of the peptidoglycan-linked cadaverine, were investigated on the assumption that cadaverine associates with the structural connection between the outer membrane and the peptidoglycan, and thereby serves as the functional counterpart of MLP (Kojima et al. 2011). Interaction of the outer membrane component with the

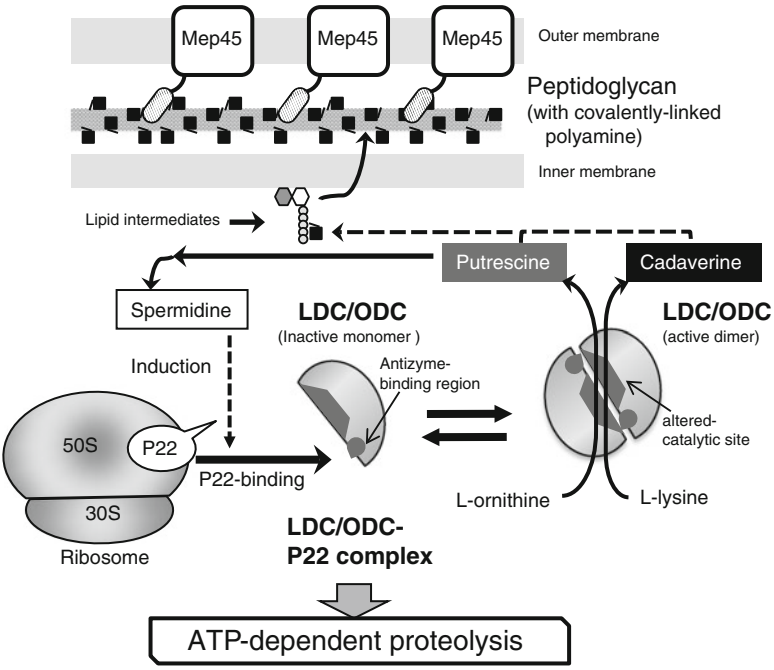


**Fig. 8.1** Model of cadaverine biosynthesis, its P22-mediated regulation, and its role in the cell wall-anchoring system in *Selenomonas ruminantium*. Refer to the text for details

peptidoglycan-linked cadaverine was examined. It was found that the major outer membrane protein Mep45, which has an *N*-terminal periplasmic surface layer homology (SLH) domain, acts as the binding partner for the peptidoglycan in a manner that is dependent on the presence of peptidoglycan-linked cadaverine (Fig. 8.1).

### *8.1.2 Novel Characteristics of* S. ruminantium *LDC/ODC*

In *S. ruminantium*, cadaverine is synthesized by decarboxylating L-lysine. In most bacteria, LDC functions mainly as an inducible enzyme that appears only under certain conditions, such as low pH. However, in *S. ruminantium*, LDC is thought to be produced constitutively, based on analyses of the enzyme activities in cells grown under various conditions (Kamio and Terawaki 1983). Its molecular mass is 43 kDa, comprising 393 amino acid residues, and it forms a homodimer of 88 kDa in the native state, differing completely from *Escherichia coli* LDC, which forms a 780-kDa decameric structure consisting of identical subunits of 80 kDa. The detailed enzymatic properties and the nucleotide sequence of the gene encoding *S. ruminantium* LDC were determined and investigated (Takatsuka et al. 1999a, 2000). An in vitro kinetic study using a purified enzyme preparation of LDC demonstrated

characteristic substrate specificity, decarboxylating L-lysine and L-ornithine, and synthesizing cadaverine and putrescine, respectively. The $K_m$ values for L-lysine and L-ornithine were 0.63 and 1.2 mM, respectively. The decarboxylating activity toward both substrates was competitively inhibited by either DFML or DL-α-difluoromethylornithine (DFMO), the specific inhibitors of LDC and ODC, respectively. Hence, LDC from *S. ruminantium* has been designated as lysine/ornithine decarboxylase (LDC/ODC), as its catalytic domain for both substrates was supposed to be located in an identical region (Takatsuka et al. 2000).

The pyridoxal-5′-phosphate (PLP)-dependent enzymes are generally classified into seven folding types based on their predicted secondary structures, and the bacterial ODCs and LDCs belong to fold type I (Grishin et al. 1995). The nucleotide sequence of deduced amino acid sequence from the gene encoding LDC/ODC shows significant homology to type III PLP-dependent ODC (cd00622 in the conserved domain and protein classification database of NCBI), which is composed mainly of eukaryotic ODC, including that of mouse, *Neurospora crassa*, *Trypanosoma brucei*, and *Caenorhabditis elegans*, rather than general bacterial LDC. The amino acid residues implicated by the fundamental enzymatic function of such domains as the PLP-binding domain, the substrate-binding domain, and the region responsible for dimer formation are all conserved in LDC/ODC (Fig. 8.2). In addition, LDC/ODC has a mammalian ODC AZ-binding region containing three amino acid residues that are pivotal for AZ binding (Fig. 8.2) (Takatsuka et al. 2000). These findings indicate that LDC/ODC resembles the eukaryotic ODC more than the bacterial one.

The eukaryotic ODC has exclusive substrate specificity toward L-ornithine. However, LDC/ODC displays dual specificity toward both L-lysine and L-ornithine. The five amino acid residues ($A^{44}$, $G^{45}$, $V^{46}$, $P^{54}$, and $S^{322}$) conferring this characteristic substrate specificity were identified based on sequence alignment with the catalytic domain of mouse ODC and experimental analysis using mutant LDC/ODC (Fig. 8.2) (Takatsuka et al. 1999b, 2000). Substituting these five residues of LDC/


**Fig. 8.2** Alignment of functional segments in *S. ruminantium* lysine/ornithine decarboxylase (LDC/ODC), related bacterial ODCs, and type III pyridoxal-5′-phosphate (PLP)-dependent ODCs. Residues with *dark and light shading* are involved in PLP binding and dimerization, respectively. Conserved substrate-binding sites are indicated by *arrows*. The amino acid residues conferring the characteristic substrate specificity of *S. ruminantium* LDC/ODC are indicated in *boxes*. The antizyme binding region in mouse ODC and the corresponding region in *S. ruminantium* LDC/ODC are *underlined*, and pivotal amino acids for antizyme binding are indicated by *asterisks*

ODC for the corresponding residues of mouse ODC (A44V/G45T/V46P/P54D/S322A) converted its substrate specificity to L-ornithine (70 times higher than wild-type LDC/ODC). According to these findings, LDC/ODC can be thought of as a type of eukaryotic ODC, but containing the altered catalytic domain conferring the characteristic L-lysine substrate specificity, which enables cadaverine production. This feature is quite distinct from the reported bacterial LDCs or ODCs (Takatsuka and Kamio 2004).

## 8.2 P22-Mediated Regulation of LDC/ODC Biosynthesis in *Selenomonas ruminantium*

### *8.2.1 Discovery of P22, a Direct Counterpart to Mammalian ODC Antizyme, and Its Characteristics*

Discovered a 22-kDa protein (P22), a direct counterpart to mammalian antizyme (AZ) for ODC, which is designated "bacterial antizyme" in *S. ruminantium* Yamaguchi et al. (2002). During research on the fluctuation of LDC/ODC activity in *S. ruminantium*, we noticed that LDC/ODC activity drastically decreased at the early stationary phase, and showed that this occurred because of the rapid proteolytic degradation of LDC/ODC. The half-life of LDC/ODC in the early stationary phase (10 min) was estimated to be shorter than that in the mid-log phase (60 min). This degradation was ATP dependent and was inhibited by phenylmethylsulfonyl fluoride, strongly suggesting the existence of a proteolytic regulatory process for LDC/ODC (Yamaguchi et al. 2002). This phenomenon is quite reminiscent of AZ-mediated regulation of ODC in eukaryotes, in which the AZ binds to monomeric ODC, which is in rapid equilibrium with the active dimeric form, and triggers the ATP-dependent proteolytic degradation by 26S proteasome (Hayashi et al. 1996). In fact, LDC/ODC possesses the AZ-binding region of mouse ODC (Takatsuka et al. 2000). These findings led to further investigation of the degradation mechanism of LDC/ODC. In 2002, Yamaguchi et al. (2002, 2006) isolated a 22-kDa protein (P22) as a stimulating factor for the ATP-dependent proteolysis of LDC/ODC from putrescine-grown cells. It displays no direct degradation activity toward LDC/ODC, but binds to the AZ-binding region of LDC/ODC and triggers ATP-dependent proteolysis (Fig. 8.1). This proteolytic system was completely compatible with the factors implicated in the AZ-mediated regulatory systems of mouse ODC, such as mouse AZ and 26S proteasome, and P22 acts as an exact counterpart of eukaryotic AZ. Mouse AZ can bind to the AZ-binding region of LDC/ODC and triggers proteolysis. The 26S proteasome can imitate LDC/ODC, whether triggered by P22 or mouse AZ. Yamaguchi et al. (2006) showed that MG132, a proteasome ubiquitination inhibitor, clearly inhibited the degradation of LDC/ODC in the presence of either P22 or AZ in rabbit reticulocyte lysate. These results demonstrate that degradation of LDC/ODC is catalyzed by proteasomes and is dependent on AZ or P22 without ubiquitination.

The $K_D$ (M) values of P22 and mouse AZ for LDC/ODC were $8.55 \times 10^{-11}$ and $10.8 \times 10^{-11}$, respectively. However, neither P22 nor mouse AZ bound to the *S. ruminantium* LDC/ODC mutant in the mouse AZ binding region (see Sect. 8.1.2), in which three Lys residues (K103, K123, and K126) were replaced by Ala, resulting in null degradation of the mutant LDC/ODC in the cell-free system. This tight interaction between the enzyme and AZ should prevent the re-association of the LDC/ODC subunits into the active homodimer. These results suggest that the initial signal in LDC/ODC degradation may actually be a subtle conformational change in the enzyme that occurs by P22 binding to the enzyme and may subsequently facilitate its degradation. Interestingly, the C-terminus of *S. ruminantium* LDC/ODC is shorter than those of the ODCs from mouse and yeast. Compared with mouse ODC, the *S. ruminantium* LDC/ODC lacks 31 amino acids of the C-terminus (Takatsuka et al. 2000). This region of mammalian ODC has been shown to be essential for the extremely fast turnover of the protein (Ghoda et al. 1989). However, LDC/ODC is degraded rapidly in the presence of AZ or P22 in rabbit reticulocyte lysate, as well as in the *S. ruminantium* degradation system, showing that P22 fulfills an AZ-like role in the rabbit reticulocyte lysate. Interestingly, the five residues of the carboxyl-terminal of LDC/ODC, and those of a transposition repressor protein (Rep) of a bacteriophage Mu consisting of 196 amino acid residues, are –KKAAV$^{393}$ and –VKKAV$^{196}$, respectively. In *E. coli*, Rep is resistant to ATP-dependent ClpXP protease, whereas RepV196A, a mutant of Rep in which the C-terminal Val residue is replaced by an Ala residue, is easily digested by ATP-dependent ClpXP protease (Batty and Nakai 2008).

The nucleotide sequence of the gene encoding P22 is entirely different from that of mouse AZ (Yamaguchi et al. 2006) but indicates that P22 is a ribosomal protein L10 of this bacterium. In fact, P22 was detected as the constituent of the 50S subunit of the ribosome (Yamaguchi et al. 2006). Based on the genome-sequence analysis of *S. ruminantium*, there is no other putative L10 protein except besides P22. Therefore, it is expected that P22 plays dual roles, both as the ribosomal L10 protein and as the antizyme. There is no report regarding such a regulatory system involving ribosomal protein serving as the bacterial antizyme. However, whether P22 is immediately assembled as a ribosomal unit within the mature ribosome, or requires dissociation from the ribosome to fulfill its AZ function, the molecular mechanism for the switching of the P22 that can act as AZ remains unknown. Yamaguchi et al. (2008) indicated that the 79 C-terminal residues are involved in the AZ function of P22 (Fig. 8.3a). The crystal structure of the L10/L12 N-terminal domain complex of *Thermotoga maritima* revealed that the C-terminal region of the L10 protein in the bacterial 50S ribosome forms a complex with L7/12 proteins to produce a stalk structure (Diaconu et al. 2005). It is presumed that the C-terminal of L10 should be free from L7/12 protein when L10 interacts with LDC/ODC as antizyme.

Interestingly, the L10 protein of *E. coli* has no AZ activity similar to P22, and it was determined that two segments specifically existing in the C-terminal region of P22 (K$^{101}$NKLD$^{105}$ and G$^{160}$VIRNAVYVLD$^{170}$) are responsible for binding to LDC/ODC

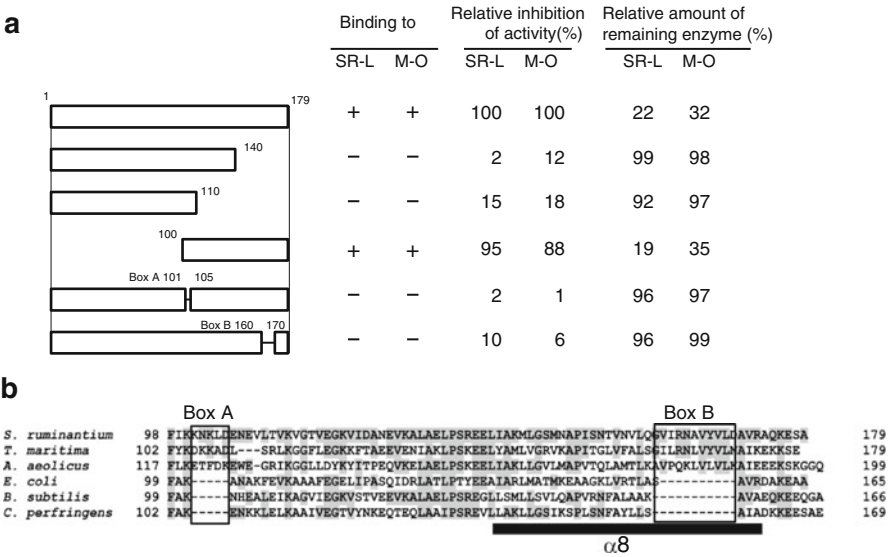


**Fig. 8.3** **a** Schematic representation of *S. ruminantium* P22 protein and its mutants. Their activities to LDC/ODC are also indicated. *SR-L* and *M-O* represent *S. ruminantium* LDC/ODC and mouse ODC, respectively. **b** Amino acid sequence alignment of the C-terminal region of P22 with other prokaryotic L10 proteins. Amino acids identical to P22 are *shaded*. The **α**8 helix region involved in the stalk structure of L7/12 proteins in the 70S ribosome is indicated by the *thick bar*. *Box A* and *Box B* indicate segments crucial for binding to LDC/ODC in P22

(Fig. 8.3b) (Yamaguchi et al. 2008). The insertions of the segments in corresponding regions were also found in *T. maritima* and *Aquifex aeolicus* L10 proteins.

### 8.2.2 *Correlation Between Appearance of P22 in the Cytoplasm and the LDC/ODC Degradation During the Growth of* S. ruminantium *Cells*

The dramatic appearance of P22 occurred in the cytoplasm from *S. ruminantium* that was grown with putrescine at the early stationary phase, and thereafter P22 decreased within 1 h. This rapid degradation of P22 accompanies LDC/ODC degradation and is caused by the ATP-dependent protease. Interestingly, a marked accumulation of spermidine but not of putrescine was found in putrescine-grown cells at the early stationary phase, suggesting that P22 should be induced by spermidine in the early stationary phase. This accumulation was closely linked to the initiation of LDC/ODC degradation (unpublished data). Intact P22 (=L10) acts as an AZ, and a ribosomal frameshift for the expression of alternate protein is not required, whereas production of AZ in mammalian cells requires a specific +1 translational frameshift.

## 8.3 Phylogenetic Distribution of LDC/ODC and Ribosomal L10

*Selenomonas ruminantium* LDC/ODC shows eukaryotic ODC-like properties with respects to both the primary sequence homology and the enzymatic characteristics, representing a phylogenetically intriguing feature.

The homologues of *ldc*/*odc*, having the conserved amino acids of type III PLP-dependent ODC, are well distributed among the *Veillonellaceae* family of bacteria. Therefore, the existence of the eukaryotic type of ODC may represent a distinguishing feature of this family. In addition, the ODCs of *A. aeolicus* and *T. maritima* are classified as PLP-dependent ODCs (Fig. 8.2). The L10 proteins of these bacteria contain two segments that are demonstrated in P22 to be responsible for binding to LDC/ODC (Yamaguchi et al. 2008) (Fig. 8.3). Unfortunately, the AZ-binding regions of *A. aeolicus* and *T. maritima* ODCs are incomplete, and they possess C-terminal amino acid sequences distinct from that of Mu Rep protein.

Hence, the potential of L10 (=P22) for AZ-mediated regulatory processes requires further research.

## 8.4 Concluding Remarks

Research into the "cadaverine physiology" in *S. ruminantium* has revealed that cadaverine exerts its function in a quite distinctive way compared with other bacteria. The physiological significance of cadaverine is to mediate the structural linkage between the outer membrane and the peptidoglycan, thereby maintaining the envelope integrity. Cadaverine is synthesized in a completely eukaryotic manner, including AZ-mediated regulation (Fig. 8.3). These findings clearly indicate the biological diversity of this bacterium beyond the preexisting knowledge related to polyamine physiology, cell envelope architecture, and the enzyme regulatory system.

Further understanding of cadaverine physiology in *S. ruminantium* will expand our knowledge in this field. The major remaining challenges include isolation and characterization of the ATP-dependent protease(s) implicated in the bacterial antizyme-mediated proteolysis of LDC/ODC. This step will enable the complete in vitro reconstitution of the bacterial antizyme-mediated proteolytic system, or in other words, the "bacterial proteasome," and lead to further understanding of the molecular mechanism of this system.

**Acknowledgments** We are grateful to all those who have supported us in our recovery and continued research following the crisis that occurred on 11 March 2011. We wish to express our sincere and heartfelt concern for the people still suffering as a result of the disaster, and wish them full and early recovery with fondest regards. We are also grateful to the director of the Center for Academic Publications Japan for permission to reproduce some sentences from the following paper: *Journal of Nutritional Science and Vitaminology* 58:153–156 (2012) by S. Kojima and Y. Kamio. Our works cited in this article were supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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# Chapter 9
# Polyamine Homeostasis in Plants: The Role(s) of Evolutionarily Conserved Upstream ORFs

**Sunil Kumar Thalor, Thomas Berberich, and Tomonobu Kusano**

**Abstract** Cellular polyamine (PA) concentrations are strictly controlled by complex regulatory processes that occur during the synthesis, catabolism, and transport of this compound. These processes include translational repression mediated by upstream open reading frames (uORFs) found in the mRNAs of genes involved in PA metabolism. First, we discuss the roles of dual uORFs in the *S*-adenosylmethionine decarboxylase gene. Second, we summarize the role of the fourth uORF in a basic helix-loop-helix transcription factor gene, *SAC51*, in thermospermine action in terms of its effect on xylem vessel differentiation and stem growth. Third, we discuss the sequence-conserved uORFs found in polyamine oxidase (*PAO*) transcripts encoding peroxisome-localized PAOs. It is currently unclear whether these uORFs cause the repression of PAO translation. Finally, because uORF-mediated repression of arginine decarboxylase (ADC) translation was reported in carnation, we critically assess the role of uORF in ADC translation.

**Keywords** Acaulis5 • Arginine decarboxylase • Feedback regulation • Homeostasis • Polyamine oxidase • SAMDC • Thermospermine • Translational control • Upstream open reading frame

## 9.1 Introduction

The structure of mRNA includes elements that can regulate gene expression, such as upstream open reading frames (uORFs), which are often present in the 5′-leader of the mRNA (Gallie 1993; Vaughn et al. 2012). These uORFs are defined by a start codon in the 5′-leader that is out of frame with the main open reading frame (ORF) (Calvo et al. 2009). Posttranscriptional regulation is often controlled by uORFs in

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the 5′-leaders of mRNAs. Although uORFs occur in 20 % to 50 % of eukaryotic transcripts, those encoding conserved peptides are relatively rare (fewer than 1 %) in angiosperms (Hayden and Jorgensen 2007). To date, four types of uORFs with conserved deduced amino acid sequences have been reported in plants, all of which are involved in the translational regulation of *Arabidopsis thaliana* genes. These genes include the following: (1) the group *S* basic region leucine zipper protein-encoding gene *bZIP11*, which is involved in the sucrose response (Rook et al. 1998; Wiese et al. 2004; Thalor et al. 2012); (2) the *S*-adenosylmethionine decarboxylase (*SAMDC*) gene, which is required for polyamine biosynthesis (Hanfrey et al. 2002, 2003, 2005; Ivanov et al. 2010); (3) *SAC51*, which encodes a basic helix-loop-helix-type transcription factor that suppresses the auxin-mediated xylem vessel differentiation pathway; and (4) *NMT1*, encoding a phosphoethanolamine *N*-methyltransferase, which is involved in phosphocholine biosynthesis (Tabuchi et al. 2006). *ACL5* encodes thermospermine (T-Spm) synthase in *Arabidopsis*, and its loss-of-function mutant (*acaulis5*, abbreviated as *acl5*) displays stunted growth in stems. One (*sac51-d*) of the *suppressor of acaulis* (*sac*) mutants that recover normal stem growth has a premature stop codon in the fourth uORF of the five uORFs in the aforementioned *SAC51* transcript (Imai et al. 2006; Takano et al. 2012). It should be noted that two of the four genes listed here are involved in polyamine metabolism or action. Furthermore, bioinformatic analyses have revealed that there are 27 groups of sequence-conserved uORFs in *Arabidopsis* and rice (Hayden and Jorgensen 2007; Jorgensen and Dorantes-Acosta 2012; Takahashi et al. 2012), including some polyamine oxidase (*PAO*) genes (Moschou et al. 2008; Takahashi et al. 2010; Fincato et al. 2011). In addition, Chang et al. (2000) reported that a uORF located upstream of the arginine decarboxylase (*ADC*) gene in carnation represses *ADC* translation. Here, we discuss current understanding about the roles of uORFs that are positioned upstream of four PA and PA-related genes, that is, *SAMDC*, *SAC51*, *PAO*, and *ADC*.

## 9.2 *SAMDC* Gene

Putrescine (Put) is converted into triamine spermidine (Spd), which is further converted into tetraamine, spermine (Spm), or T-Spm through the transfer of an aminopropyl residue donated from decarboxylated *S*-adenosylmethionine (dcSAM). This dcSAM is generated from *S*-adenosylmethionine (SAM), which is catalyzed by SAM decarboxylase (SAMDC). Mammalian *SAMDC* transcripts contain a small ORF upstream of the main ORF encoding the hexapeptide MAGDIS. *SAMDC* is regulated at the translational level in response to cellular polyamine concentrations through interactions between the uORF-encoded peptide and polyamines. This interaction results in ribosomal stalling near the stop codon of the uORF (Ruan et al. 1996). Plant *SAMDC* mRNA has an unusually long 5′-leader. In its mRNA region, there are two conserved, overlapping uORFs (Franceschetti et al. 2001). The first uORF (tiny uORF) distal to the 5′-end is 3 to 4 codons long; the second uORF (small uORF) encodes a 48- to 55-amino acid peptide. The small uORF-encoded

peptide sequence is conserved among different plant species (Hanfrey et al. 2002, 2003, 2005) and is responsible for translational repression of the main ORF in the presence of excess polyamine; if the tiny uORF is recognized by the scanning small ribosomal subunit preinitiation complex, the small uORF is not translated, and the ribosome reinitiates translation at the start codon of SAMDC, the main ORF (Hanfrey et al. 2005; Kusano et al. 2008; Ivanov et al. 2010). Interestingly, the configuration of this tiny/small uORF, with a one-nucleotide overlap, is highly conserved from *Chlamydomonas reinhardtii*, to *Physcomitrella patens*, to higher plants (Kusano et al. 2008).

## 9.3 *SAC51* Gene

T-Spm, a structural isomer of Spm, is formed from Spd by T-Spm synthase, which is encoded by *ACL5* in *Arabidopsis* (Knott et al. 2007; Kakehi et al. 2008; Naka et al. 2010). As the *acl5* mutant is T-Spm deficient and shows severely retarded stem growth, possibly because of the overproliferation of xylem vessels, T-Spm plays a physiologically significant role in stem growth (Hanzawa et al. 2000). *ACL5* mRNA levels are reduced by the application of T-Spm, which indicates that *ACL5* expression is negatively controlled by T-Spm (Kakehi et al. 2008). The allelic mutant of *ACL5*, *tkv* (*thickvein*), also has a dwarf phenotype and thick veins in its leaves, as well as impaired polar auxin transport activity in its inflorescence stem (Clay and Nelson 2005). *ACL5* is specifically expressed in the xylem vessel elements. In wild-type *Arabidopsis*, pitted xylem vessels predominate, whereas in *acl5*, the xylem vessels are mostly of the spiral type, and pitted vessels are absent, indicating that T-Spm-deficient plants are severely affected in xylem specification (Muñiz et al. 2008). The authors further showed that ACL5 prevents premature death of developing vessel elements (Muñiz et al. 2008; Vera-Sirera et al. 2010). Takahashi and colleagues isolated four *suppressor of acaulis* (*sac*) mutants to unveil the link between T-Spm and xylem differentiation (Imai et al. 2006). One of the *sac* mutants, *sac51-d*, has a mutation in the fourth of five uORFs found in the 5′-leader of the *SAC51* mRNA. This fourth uORF is evolutionarily conserved. The *sac51-d* mutation leads to stimulated *SAC51* translation, indicating that the conserved intact uORF represses *SAC51* translation. A hypothetical model to explain how T-Spm determines auxin-induced xylem differentiation has also been proposed (Vera-Sirera et al. 2010; Takano et al. 2012; Yoshimoto et al. 2012).

## 9.4 *PAO* Gene

Translational control of polyamine catabolism has been reported in mammals (Ivanov et al. 2010; Perez-Leal and Merali 2012). Spermidine/spermine $N^1$-acetyltransferase (SSAT) is the rate-limiting enzyme for PA catabolism and is

required before PAO action in mammals. Most vertebrate *SSAT* mRNAs contain uORFs. For example, in the human *SSAT* transcript, uORF1 encodes four amino acids and is separated from the downstream uORF2 by 28 nucleotides. The uORF2 encodes a putative protein of 44 amino acids (Ivanov et al. 2010). However, the involvement of the uORFs in translational control of *SSAT* has not been supported by other studies. Two independent groups showed that the translational repression of *SSAT* is highly unusual because it does not involve the 5′-or 3′-UTRs (Parry et al. 1995; Butcher et al. 2007). Recently, an interesting and important control mechanism involving the release of a translational repressor protein (nucleolin) that interacts with the 5′-end of the *SSAT*-ORF has been proposed (Perez-Leal et al. 2012).

*SSAT* does not exist in plants, and thus this type of acetyl modification is not required for plant PAO-mediated catabolism. We have noticed that some plant *PAO* genes contain uORFs whose sequences are conserved in a variety of plant species (Fig. 9.1a). The uORFs encode 47- to 74-amino-acid peptides, and their amino-terminal sequences, including approximately 30 amino acids, are highly conserved (Fig. 9.1b). The peroxisomal localization of OsPAO3, AtPAO2, and AtPAO3 has been confirmed experimentally (Moschou et al. 2008; Kamada-Nobusada et al. 2008; Ono et al. 2012). The other members of this family are also expected to localize to peroxisomes, because all carry peroxisome-targeting signals (either SRL or SRM) at their carboxy-termini. This observed conservation suggests that these proteins play important roles; however, this topic has not been well investigated. Recently, Guerrero-González et al. (2014) reported that the uORF of *AtPAO2* transcript negatively controls the translation of the *AtPAO2* main ORF and that PA participates in this control. This observation could be confirmed for *AtPAO2* while the uORF of *AtPAO3* transcript exhibits the opposite effect on the translation of the *AtPAO3* main ORF (TK, unpublished data). Thus, the possibility that the sequence-conserved uORFs in *PAO* genes play different roles in translational regulation is highly unlikely. Except for *AtPAO3* and *HvPAO* (Fig. 9.1a), the positional relationship in the coding frame between the uORF and main PAO ORF differs by +1. An attempt to detect the +1 frameshifted product, a uORF and PAO ORF fusion, in the absence or presence of various PAs, was unsuccessful (T. Kusano, unpublished data). Therefore, further investigation of the physiological role(s) of the uORFs in *PAO* transcripts is warranted.

## 9.5 *ADC* Gene

Do the uORFs present in some *ADC* transcripts repress the translation of the main ORF in the gene? Using a carnation *arginine decarboxylase* gene (*DcADC*, which refers to the *Dianthus caryophyllus* L. *ADC* gene), Chang et al. (2000) showed that the *DcADC* transcript contains a short uORF encoding a 7-amino-acid peptide (MQKSLHI) and that this uORF-encoded peptide represses the translation of the downstream ORF. Several plant *ADC* genes contain uORFs; for example, in *Arabidopsis* and rice, of the two *ADC*s, *ADC2* transcripts contain uORFs but *ADC1* do not. The deduced amino acid sequences encoded by the uORFs from *Arabidopsis*

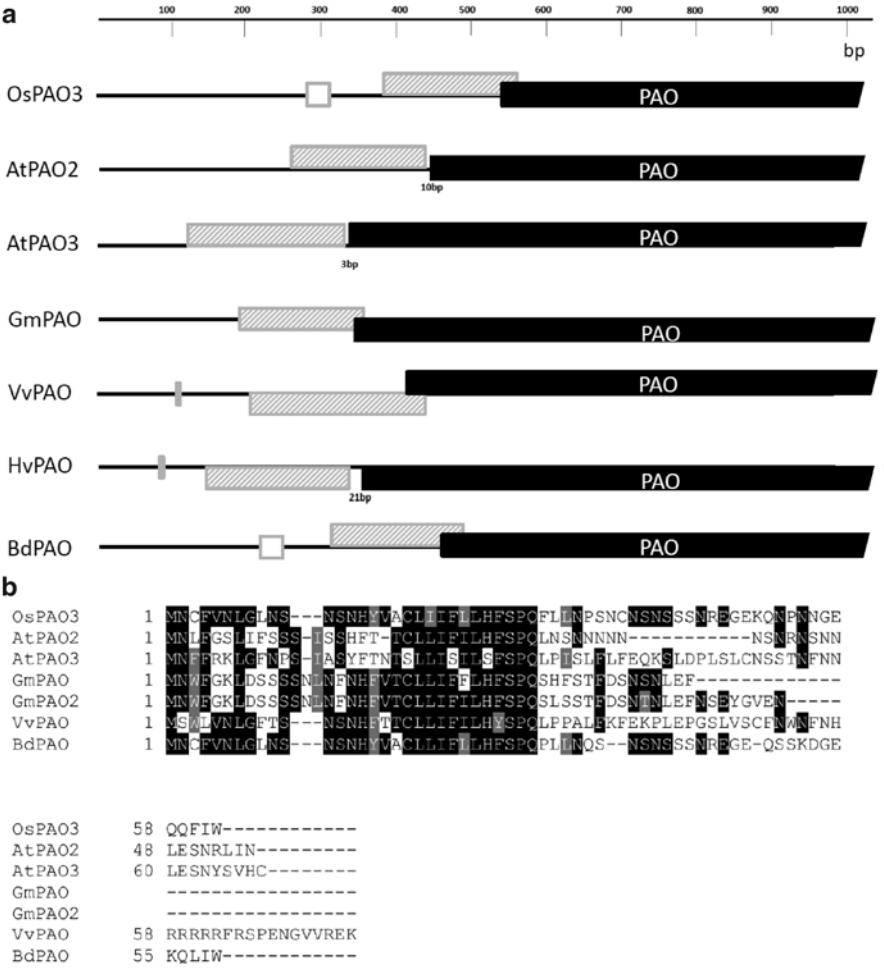


**Fig. 9.1** Schematic diagram of some plant polyamine oxidase (*PAO*) transcripts and alignment of the deduced amino acid sequences from sequence-conserved upstream open reading frames (uORFs). **a** Positional relationship of uORF(s) and main ORF(s) in some plant *PAO* transcripts. *Solid black line*, 5′ leader region; *gray-lined box*, uORF; *gray-lined box* with crossed-lines, sequence-conserved uORF; *black box*, PAO-encoded ORF. Position of boxes indicates the ORF that begins at the first frame (above the *solid line*), second frame (on the *solid line*), and third frame (beneath the *solid line*), respectively. For example, in the case of OsPAO3, the sequence-conserved uORF and the main PAO ORF start from the first and second frame, respectively. The genes IDs or accession numbers are as follows: OsPAO3 (NM_001060458, Os04g0623300), AtPAO2 (At2g43020), AtPAO3 (At3g59050), GmPAO (*Glycine max*, XM_003551900), GmPAO2 (*Glycine max*, XM_003532021), VvPAO (*Vitis vinifera*, XM_002282934), HvPAO (Hordeum vulgare, AK368797), and BdPAO (*Brachypodium distachyon*, XM_003580531). **b** Amino acid sequence alignment of the deduced amino acid sequences from sequence-conserved uORF(s) found in PAOs

and rice *ADC2*s are MVGRIC and MLPHKH, respectively, and thus no homology is observed among these putative gene products, including that of *DcADC*. Furthermore, the predicted amino acid sequences of the uORFs from other plant

*ADC*s are also not conserved and vary in length from 4 to 11 amino acids (Peremarti et al. 2010). The following data appear to contradict the notion that uORFs located upstream of the main ORFs of *ADC* are involved in its translational repression. First, rice *OsADC1* is expressed in leaves, roots, and stems, while the expression of *OsADC2* is restricted to stem tissue (Peremarti et al. 2010). The same research group showed (via an antisense method) that rice plants with reduced *ADC* mRNA levels contain significantly reduced levels of Put and Spd, whereas the levels of ornithine decarboxylase (*ODC*), *SAMDC*, and spermidine synthase (*SPDS*) transcripts are not affected in these plants (Trung-Nghia et al. 2003). These results indicate that the endogenous PA biosynthetic pathway is uncoupled from manipulated PA levels. Second, posttranscriptional and PA-responsive feedback regulation of *ADC* or *ODC* is not observed in tobacco BY-2 cells (Illingworth and Michael 2012). Third, the uORF in *Arabidopsis ADC2* does not repress the translation of the main ORF (Sho Izawa, unpublished data).

## 9.6 Conclusion

In vertebrate cells, several genes involved in PA metabolism, including the antizyme gene, are equipped with a uORF-mediated translational regulation mechanism. *SAMDC* has a similar mechanism in plants, but it has a distinct main ORF translational control mechanism involving dual uORFs. *SAC51* translation is repressed by the sequence-conserved uORF in the 5′-leader region of the *SAC51* transcript. This uORF-mediated repression of *SAC51* translation is deregulated by T-Spm. This fine-tuned regulatory mechanism contributes to auxin-induced xylem differentiation. The uORF found in the *ADC* gene was not conserved during evolution. Experimental and circumstantial data do not support the idea that this uORF may repress *ADC* translation. To explore the role(s) of sequence-conserved uORFs in some *PAO* members, future research should focus on which products may be located in peroxisomes.

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# Part IV
# Roles of Polyamines in Growth

# Chapter 10
# Role of the Polyamine Spermidine as a Precursor for Hypusine Modification in eIF5A

**Edith C. Wolff and Myung Hee Park**

**Abstract** Although polyamines exert various effects on nucleic acids and macromolecular synthesis as polycations, spermidine is covalently incorporated into a single protein, eukaryotic initiation factor 5A (eIF5A), through a unique posttranslational modification. In this reaction, the aminobutyl moiety of spermidine is conjugated to a specific lysine residue of eIF5A to form an unusual amino acid, hypusine [$N^{\varepsilon}$-(4-amino-2-hydroxybutyl)-lysine]. It occurs by two enzymatic steps catalyzed by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH). Hypusine synthesis occurs exclusively in eIF5A and is essential for eukaryotic cell proliferation. Although only a small percentage of the total spermidine in cells is used for hypusine formation, cells cannot survive/grow when hypusinated eIF5A falls below a critical level. Inactivation of the *eIF5A* gene or *DHS* gene is lethal in yeast and in mouse, further indicating the vital role of hypusinated eIF5A. eIF5A has been proposed to promote translation of a subset of cellular mRNAs. Indeed, recent evidence suggests that eIF5A facilitates translation at the elongation step, particularly at multiple strings of proline residues. A model of eIF5A docked in the ribosome reveals the hypusine directed toward the peptidyl transferase center. Thus, the hypusine modification defines a link between polyamines and cell growth, through promotion of translation.

**Keywords** Deoxyhypusine hydroxylase (DOHH) • Deoxyhypusine synthase (DHS) • eIF5A • Hypusine • Spermidine • Translation

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## Abbreviations

| | |
|---|---|
| DHS | Deoxyhypusine synthase |
| DOHH | Deoxyhypusine hydroxylase |
| EF-P | Bacterial elongation factor P |
| eIF5A | Eukaryotic initiation factor 5A |
| GC7 | $N^1$-guanyl-1,7-diaminoheptane |
| SSAT1 | Spermidine/spermine acetyltransferase 1 |

## 10.1 Introduction

The polyamines, putrescine, spermidine and spermine, are ubiquitous natural compounds that are required for eukaryotic cell growth and survival. With their primary and secondary amino groups protonated at physiological pH, these polycations interact with negatively charged macromolecules such as DNA, RNA, proteins, and phospholipids and influence their activities. Besides these polycationic functions, the polyamine spermidine is required as a donor of its butyl amine moiety in the posttranslational formation of hypusine in eukaryotic translation initiation factor 5A (eIF5A) (Scheme 10.1). This process converts an inactive eIF5A precursor to an active protein, a factor essential for protein synthesis and cell growth. Hypusine synthesis thus represents an indispensable function of polyamines in cell growth. In this chapter, we describe briefly the discovery of hypusine, identification of the hypusine-containing protein, biosynthetic pathway of hypusine and its inhibitors, and discuss the function, regulation, and mechanism of eIF5A in translation and cell proliferation. Because of space limitations, topics on the role of eIF5A isoforms in cancer [reviewed elsewhere (Caraglia et al. 2013; Park et al. 2014; Wang et al. 2013)] and other diseases, such as AIDS and diabetes, are not covered, and only a selection of references are given.

### *10.1.1 Hypusine and Its Biosynthesis*

A modified lysine, hypusine [$N^{\varepsilon}$-(4-amino-2-hydroxybutyl)-lysine], named for its structural relationship to hydroxyputrescine and lysine, was first isolated from bovine brain extracts and the structure determined (Shiba et al. 1971). It was found to occur in all animal tissues, as the free amino acid as well as protein-bound form (Nakajima et al. 1971). In 1981, in lymphocytes cultured in a medium containing radioactive spermidine, one specific protein was radiolabeled. Hypusine was discovered to be a component of this labeled protein (Park et al. 1981), which was later identified as an eukaryotic translation initiation factor 4D (eIF-4D, current nomenclature, eIF5A) (Cooper et al. 1983). eIF5A and hypusine exist in all eukaryotes,

including yeast (for reviews, see Chen and Liu 1997; Park et al. 1993; Park 2006). Hypusine and its precursor, deoxyhypusine, also occur in Archaebacteria, but not in Eubacteria. Hypusine is formed only posttranslationally; thus, free hypusine in urine or tissue is presumed to be derived from the breakdown of eIF5A.

The biosynthesis of hypusine (Scheme 10.1) is catalyzed by two specific enzymes, deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) (Park 2006; Wolff et al. 2007). DHS catalyzes an NAD-dependent cleavage of spermidine with the transfer of its 4-aminobutyl moiety to the terminal N of a specific lysine of eIF5A (Lys 50 in human, Lys 51 in yeast) to form an intermediate, deoxyhypusine residue. Importantly, neither putrescine nor spermine can substitute for spermidine as a substrate, emphasizing the critical role of spermidine in cell growth.

The second enzyme, DOHH, irreversibly adds a hydroxyl group to the side chain of deoxyhypusine (Park et al. 2006; Park 2006). Like DHS, it is entirely specific for its protein substrate. It is an Fe(II)-dependent monooxygenase with a superhelical structure and a reaction mechanism distinct from other known protein hydroxylases (Kim et al. 2006b).

### *10.1.2 Additional Posttranslational Modifications in eIF5A, Acetylation and Phosphorylation*

Hypusine synthesis activates eIF5A and directs it into the cytoplasmic compartment (Lee et al. 2009). Localization of hypusinated eIF5A in the cytoplasm and its association with ribosomes is critical for its role in translation. Normally, eIF5A undergoes hypusine modification immediately after its translation, and this process is irreversible. Because of this irreversibility and the long half-life of eIF5A, it may be difficult to modulate the activity of hypusinated eIF5A rapidly. In a search for additional, reversible posttranslational modifications, it was found that eIF5A can be acetylated at two conserved lysine residues, K47 (Klier et al. 1995) and K68 (in the human sequence) (Kim et al. 2006a) and also at the hypusine residue (Lee et al. 2009, 2011). Acetylation of eIF5A at Lys47 by histone acetyltransferase, PCAF, (Ishfaq et al. 2012) (Scheme 10.1), would render eIF5A inactive (Cano et al. 2008), and directs it into the nuclei (Ishfaq et al. 2012). eIF5A can also be inactivated by acetylation at the hypusine residue by the spermidine/spermine acetyltransferase 1 (SSAT1) (Scheme 10.1) (Lee et al. 2011). Although such acetylation can be demonstrated in vitro and in cells, cellular levels of acetylated eIF5A are normally quite low, and their accumulation may become significant only upon induction of acetylating enzymes or inhibition of deacetylating enzymes.

eIF5A proteins from *Saccharomyces cerevisiae*, *Trichomonas vaginalis*, and maize also undergo phosphorylation on specific Ser or Thr residues. Ser2 phosphorylation of maize eIF5A was shown to cause its sequestration in the nucleus. The significance of the nuclear accumulation of acetylated or phosphorylated eIF5A is unknown.

## 10.2 The Role of eIF5A in Cell Growth

A critical role for eIF5A in cell growth was first suggested by the observation that the hypusine-containing protein (later identified as eIF-4D/eIF5A) dramatically increased in lymphocytes upon activation with a mitogen (Cooper et al. 1983; Park et al. 1981). Hypusine-containing protein was found in other mammalian cells and a correlation between the rate of hypusine synthesis and growth was confirmed in rat hepatoma tissue culture cells (Gerner et al. 1986) and in NIH3T3 cells upon serum stimulation (Chen and Chen 1997b). Moreover, the hypusine synthesis rate was significantly elevated in *Ras* oncogene-transfected NIH3T3 cells compared to untransfected NIH3T3 cells (Chen and Chen 1997b). In contrast, hypusine synthesis was markedly reduced in human fibroblast cells undergoing senescence (Chen and Chen 1997a).

Convincing evidence for the essential role of eIF5A and hypusine modification on cell growth was derived from gene inactivation studies in yeast *S. cerevisiae* and mouse. Disruption of both *eIF5A* genes (Schnier et al. 1991) or a single *DHS* gene (Park et al. 1998; Sasaki et al. 1996) causes growth arrest and loss of viability in yeast. Similarly, inactivation of the *eIF5A* gene or the *DHS* gene in mouse leads to embryonic lethality at the early stage of gestation (E6.5) (Nishimura et al. 2012). The hydroxylation step of hypusine synthesis is not essential in yeast, as the *DOHH* deletion strain is viable. However, in higher eukaryotes, the second step appears to be important, because *DOHH* mutation leads to growth and developmental defects in *Drosophila* (Patel et al. 2009).

As the in vivo polyamine functions in cell growth have remained obscure for decades, it was questioned whether hypusine formation represents the main or the sole factor in the polyamine requirement in eukaryotic cells. In yeast *S. cerevisiae*, it seems to be the case, because a mutant strain deficient in spermidine biosynthesis can grow at a nearly normal rate with <0.2 % of spermidine, consuming up to 54 % of cellular spermidine for hypusine synthesis (Chattopadhyay et al. 2008). In mammalian cells, the question was addressed by depletion of spermidine with inhibitors of polyamine biosynthesis and supplementation with spermidine analogues. In L1210 cells depleted of spermidine by an inhibitor of *S*-adenosylmethionine decarboxylase, only those closely related spermidine analogues that could serve as the substrate for DHS could support long-term growth in the absence of natural spermidine (Byers et al. 1992, 1994), indicating that hypusine synthesis is a core element of the polyamine requirement. Consistent findings were obtained in DU145 prostate cancer cells depleted of spermidine by treatment with α-DFMO (α-difluoromethyl ornithine), an irreversible inhibitor of ornithine decarboxylase (Hyvonen et al. 2007). In this study, the acute phase of cytostasis (within 6 days of α-DFMO treatment) could be reversed by all methylated analogues of spermidine and spermine, but long-term growth (>9 days) could only be supported by those analogues that can serve as a precursor for hypusine synthesis. These findings further suggest two elements of polyamine function in cells: the first, a polycationic function that can be fulfilled by various analogues of spermidine, and spermine, and the second, the function of supporting hypusine synthesis that requires a close structural similarity to spermidine. These two independent aspects of polyamine function in mammalian cell growth were also suggested in an independent study that showed growth inhibition of FM3A

cells upon partial depletion of spermidine and spermine by treatment with polyamine biosynthesis inhibitors, before a decline in hypusinated eIF5A (Nishimura et al. 2005). More recently, inhibition of protein synthesis and growth was also observed in cells in which cellular spermidine and spermine were rapidly depleted by overexpression of polyamine catabolic enzyme, SSAT1, before any significant decrease in hypusinated eIF5A occurred (Mandal et al. 2013). These findings reinforce the notion that, in mammalian cells, polyamines have dual functions in promoting translation, as polycations, and as a component of hypusine in eIF5A.

## 10.3 Effects of Inhibition of eIF5A Modification

As hypusine is required for the activity of eIF5A, inhibitors were developed for inhibition of DHS and as antiproliferative agents. DHS has a narrow groove for spermidine binding, and the terminal amino groups of spermidine are anchored by the conserved acidic amino acids in the active site of the enzyme. Of many diamine and triamine derivatives tested, $N^1$-guanyldiaminoheptane (GC7) was the most potent inhibitor, with a $K_i$ value much lower than the $K_m$ for spermidine (Jakus et al. 1993). GC7 was effective in inhibiting deoxyhypusine synthesis in cells and caused cytostasis in mammalian cells (Park et al. 1994) and in various human cancer cell lines (Shi et al. 1996). It also displayed antitumor effects in an animal tumor model (Jasiulionis et al. 2007)

DOHH is a mono-oxygenase with a di-iron active center (Kim et al. 2006b; Park et al. 2006) and is inhibited by a panel of iron chelators, such as mimosine, ciclopirox[1], or deferiprone.[2] These compounds caused an arrest in cell-cycle progression at the $G_1$/S boundary, coincident with inhibition of deoxyhypusine hydroxylation (Hanauske-Abel et al. 1994). Ciclopirox inhibits endothelial cell growth and angiogenesis in vitro (Clement et al. 2002) and exerts antitumor effects in the MDA-231 xenograft in mice (Zhou et al. 2010). However, the possibility that these compounds can have other cellular targets in vivo cannot be ignored and complicates the interpretation with regard to the involvement of eIF5A.

## 10.4 The Mechanism of Action of eIF5A in Translation

eIF5A (eIF4D) was initially isolated as a factor that stimulates methionyl-puromycin synthesis, a model assay for the first peptide bond formation (Kemper et al. 1976). Although it was named as a translation initiation factor then, recent work has shown that eIF5A has a distinct effect on the elongation step of translation as measured by polysome profiles (Gregio et al. 2009; Saini et al. 2009). Dever and associates (Gutierrez et al. 2013) have reported evidence that eIF5A, similar to its bacterial orthologue EF-P (Doerfel et al. 2013; Ude et al. 2013), relieves ribosome stalling at

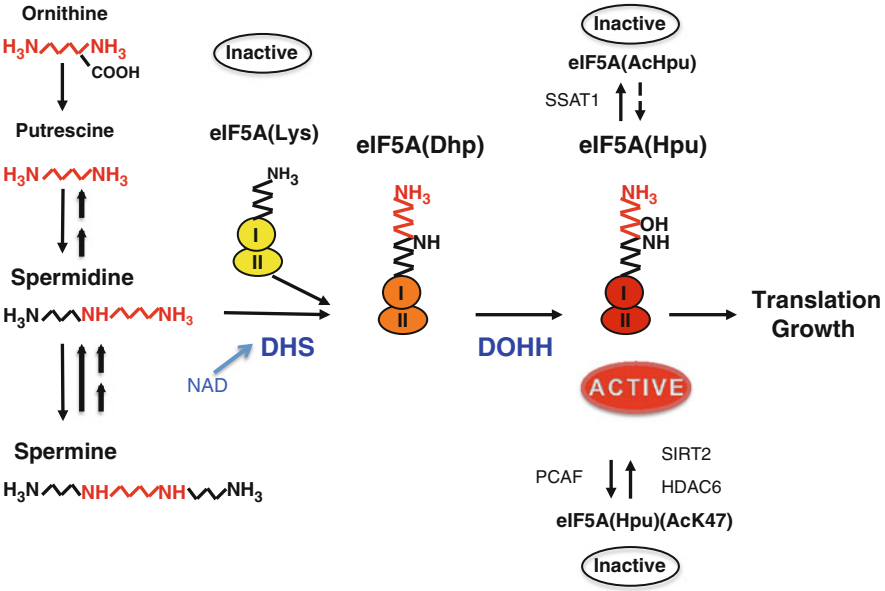
[1] Approved anti-fungal drug.

[2] Approved anti-thalassemia drug.

consecutive proline residues and thereby facilitates translation elongation of proteins containing multiple proline residues. A docking model of eIF5A bound to the translating ribosome predicts its hypusine side chain directed toward the peptidyl transferase center, consistent with its proposed function in translation elongation (Gutierrez et al. 2013). Future efforts will be directed to identify more eIF5A target motifs and to elucidate the precise contribution of polyamine-derived side chain of hypusine in the peptidyl transferase reaction.

## 10.5 Concluding Remarks

In spite of abundant genetic and biochemical evidence for the essentiality of polyamines in eukaryotic organisms, their precise function was not well understood for decades. One missing link was found with the discovery of the hypusine pathway and the role of spermidine for this modification and thereby in translation and cell growth. Since the first isolation of hypusine as a chemical entity in 1971, of eIF5A in 1976, and the identification of eIF5A as the single cellular protein containing hypusine in 1983, it has taken decades to establish its pathway (Scheme 10.1) and




**Scheme 10.1** Pathways of polyamine metabolism, hypusine synthesis and eIF5A activation/inactivation. A simplified diagram of polyamine interconversion is shown on the *left* (*vertically*) and, on the *right*, a path leading to hypusine formation on eIF5A, catalyzed by DHS and DOHH, (*horizontally*) and eIF5A acetylation (*vertically*). eIF5A(Lys) eIF5A precursor, eIF5A(Dhp) eIF5A intermediate containing deoxyhypusine, eIF5A(Hpu) eIF5A active form containing hypusine, eIF5A(AcHpu) eIF5A containing acetylated hypusine, eIF5A(Hpu)(AcK47) hypusinated eIF5A acetylated at Lys47, SSAT1 spermidine/spermine $N^1$-acetyltransferase, PCAF P300/CBP-associated factor, HDAC6 histone deacetylase 6, SIRT2 sirtuin-2

to determine its role in translation elongation, presumably on specific eIF5A target motifs, including consecutive proline residues. Although eIF5A activity in translation has been characterized biochemically, it needs to be related to cellular changes at the level of proteome and phenotypes. Many cellular functions have been proposed for eIF5A isoforms (not mentioned in this chapter for reasons of space constraints), including their roles in nuclear export, mRNA turnover/NMD (nonsense-mediated decay), actin cytoskeletal organization, cell wall integrity, cell-cycle progression, apoptosis, autophagy, and intracellular protein trafficking. eIF5A has also been implicated in pathological conditions such as cancer, inflammation, human immunodeficiency virus (HIV)1 infection, and diabetes. It is as yet unclear whether the pleotropic effects are caused by changes in the cellular proteome resulting from a dysfunction of eIF5A in translation elongation or whether eIF5A is a multifunctional protein. Future investigations are warranted to solve the mystery of action of this novel protein.

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# Chapter 11
# The Polyamine Modulon: Genes Encoding Proteins Whose Synthesis Is Enhanced by Polyamines at the Level of Translation

**Kazuei Igarashi, Yusuke Terui, and Keiko Kashiwagi**

**Abstract** Polyamines mainly exist as an RNA–polyamine complex in cells. Thus, the effects of polyamines on protein synthesis were examined in *Escherichia coli*, *Saccharomyces cerevisiae*, and mouse mammary carcinoma cells. It was found that several kinds of protein synthesis, which are involved in cell growth and viability, were stimulated by polyamines at the level of translation. We proposed that a set of genes whose expression is enhanced by polyamines at the level of translation can be classified as a "polyamine modulon." Thus far, 17 kinds of genes in *E. coli* and 4 kinds of genes in eukaryotes were identified as members of polyamine modulon. There are several mechanisms underlying polyamine stimulation of protein synthesis in *E. coli*. First, polyamines stimulated several kinds of protein synthesis when a Shine–Dalgarno (SD) sequence in the mRNAs is distant from the initiation codon AUG. Second, polyamines stimulated an inefficient initiation codon UUG- or GUG-dependent fMet-tRNA binding to ribosomes. Third, polyamines stimulated read-through of the amber codon UAG by Gln-tRNA$^{SupE}$ or +1 frameshifting at the termination codon UGA on the open reading frame. In eukaryotes, polyamines stimulated ribosome shunting during the scanning of the Met-tRNA$_i$-40S ribosomal subunit complex from the cap structure to the initiation codon AUG of mRNAs.

**Keywords** Bulged RNA • Cell growth • Cell viability • Polyamine modulon • Protein synthesis

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## 11.1 Introduction

The polyamines (putrescine, spermidine, and spermine) are essential for normal cell growth from bacteria and mammalian cells, and their contents in cells are exquisitely regulated by biosynthesis, degradation, uptake, and excretion (Igarashi and Kashiwagi 2010; Pegg 2009). Cellular polyamine contents increase in parallel with cell proliferation rate, and polyamines interact with acidic substances such as nucleic acids. To understand the physiological functions of polyamines, it is important to determine the distribution of polyamines among acidic substances in cells. The distribution of polyamines in *Escherichia coli* and rat liver was estimated from the binding constants of polyamines to macromolecules (DNA, RNA, and phospholipids) and nucleotide triphosphates, and their cellular concentrations (Miyamoto et al. 1993; Watanabe et al. 1991). Because ATP is a major nucleotide triphosphate, ATP was used as a representative of four nucleotide triphosphates. Polyamines did not bind to cytoplasmic proteins significantly. As shown in Table 11.1, most polyamines exist as a polyamine–RNA complex. In *E. coli*, approximately 32 mM putrescine and 7 mM spermidine existed at the logarithmic phase of growth. Of the total polyamine content, 90 % spermidine and 48 % putrescine existed as a polyamine–RNA complex. Because the binding of putrescine to macromolecules is weak, about 40 % putrescine existed as a free form in cells. The amount of putrescine and spermidine bound to RNA was estimated to be 3.5 and 1.4 mol/100 mol phosphate of RNA, respectively. In the rat liver, approximately 1.2 mM spermidine and 0.9 mM spermine existed, and putrescine concentration was very low. Among them, about 78 % spermidine and 85 % spermine existed as a polyamine–RNA complex. The amount of spermidine and spermine bound to RNA was estimated to be 1.0 and 1.2 mol/100 mol phosphate of RNA, respectively. It has been reported that the yeast $tRNA^{Phe}$ crystal structure includes two molecules of spermine (Quigley et al. 1978), which is close to our estimate for polyamine binding to RNA. From our estimation, it was suggested that a major part of the cellular function of polyamines may be explained through a structural change of RNA by polyamines.

**Table 11.1** Polyamine distribution in *Escherichia coli* and rat liver

| | *Escherichia coli* (pH 7.5, 10 mM $Mg^{2+}$, 150 mM $K^{+}$) | | Rat liver (pH 7.5, 2 mM $Mg^{2+}$, 150 mM $K^{+}$) | |
|---|---|---|---|---|
| Polyamine distribution | Putrescine (mM) | Spermidine (mM) | Spermidine (mM) | Spermine (mM) |
| (Total) (%) | 32.2 (100) | 6.88 (100) | 1.15 (100) | 0.88 (100) |
| Free (%) | 12.5 (38.8) | 0.26 (3.8) | 0.08 (7.0) | 0.02 (2.3) |
| DNA (%) | 3.0 (9.3) | 0.35 (5.1) | 0.05 (4.3) | 0.05 (5.7) |
| RNA (%) | 15.4 (47.9) | 6.17 (89.7) | 0.90 (78.3) | 0.75 (85.2) |
| Phospholipid (%) | 0.46 (1.4) | 0.05 (0.7) | 0.07 (6.1) | 0.04 (4.5) |
| ATP (%) | 0.84 (2.6) | 0.05 (0.7) | 0.05 (4.3) | 0.02 (2.3) |

## 11.2 Polyamine Modulon in *E. coli*

We looked for proteins whose synthesis is stimulated at the level of translation using a polyamine-requiring mutant of *E. coli* MA261. Cell growth of *E. coli* MA261 was stimulated three- to fivefold by addition of 100 μg/ml putrescine to the medium. Under these conditions, putrescine and spermidine contents in cells increased from 0 to 60 and from 0.8 to 7.0 nmol/mg protein, respectively. Thus far, we identified 17 kinds of protein synthesis that are stimulated by polyamines at the level of translation by the addition of putrescine to the medium (Igarashi and Kashiwagi 2010; Sakamoto et al. 2012; Terui et al. 2010, 2012). It was then proposed that a set of genes whose expression is enhanced by polyamines at the level of translation can be classified as a polyamine modulon (Igarashi and Kashiwagi 2010). There are several mechanisms underlying polyamine stimulation of the synthesis of various members of a polyamine modulon (Fig. 11.1). First, polyamine stimulation of protein synthesis can occur when a Shine–Dalgarno (SD) sequence in the mRNA is distant from the initiation codon AUG. Polyamines cause structural changes of a region of the SD sequence and the initiation codon AUG, facilitating formation of the initiation complex, for OppA, FecI ($\sigma^{18}$), Fis, RpoN ($\sigma^{54}$), H-NS, RpoE ($\sigma^{24}$), StpA, ribosome modulation factor (RMF), RpoZ (ω), and CpxR. Second, polyamines enhance the inefficient initiation codon UUG- and GUG-dependent fMet-tRNA binding for

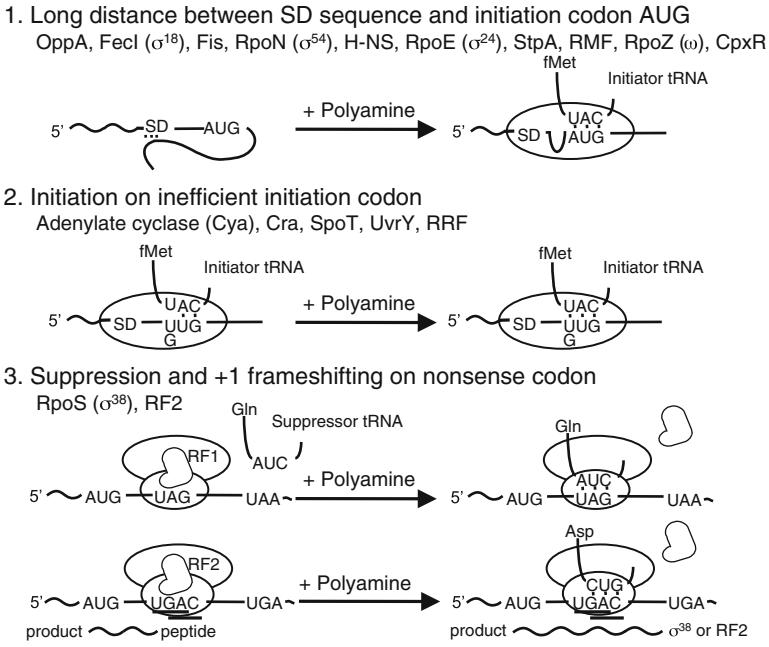


**Fig. 11.1** Three mechanisms of polyamine stimulation of protein synthesis encoded by polyamine modulon

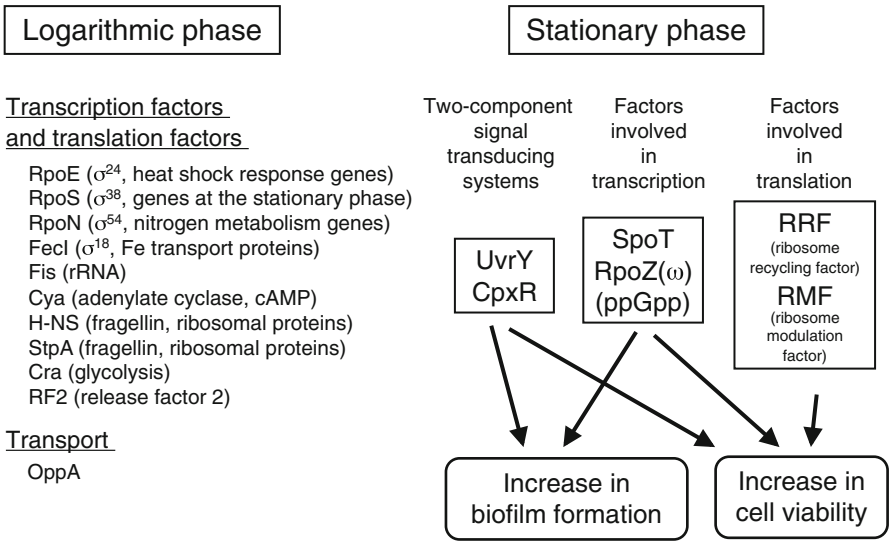


**Fig. 11.2** Functions of proteins encoded by polyamine modulon at logarithmic and stationary phases in *Escherichia coli*

adenylate cyclase (Cya), Cra, SpoT, UvrY, and ribosome recycling factor (RRF). Third, polyamines stimulate read-through by the amber codon UAG-dependent Gln-tRNA$^{SupE}$ binding to ribosome-associated *rpoS* mRNA encoding RpoS ($\sigma^{38}$), or stimulate a +1 frameshifting at the 26th UGA codon of *prfB* mRNA encoding RF2.

Functions of proteins encoded by the "polyamine modulon" were classified as shown in Fig. 11.2. Eleven kinds of proteins are related to cell growth. Among them, 10 kinds of proteins (RpoE, RpoS, RpoN, FecI, Fis, Cya, H-NS, StpA, Cra, and RF2) are involved in transcription and translation, and 1 protein (OppA) is involved in transport of nutrients. Six kinds of proteins are related to cell viability. UvrY and CpxR are response regulators in two-component signal transducing systems, SpoT and RpoZ are proteins involved in the function of ppGpp, and RMF and RRF are proteins involved in the regulation of protein synthesis.

Because nine proteins encoded by the polyamine modulon involved in cell growth are transcription factors, it is thought that synthesis of several kinds of mRNA is regulated indirectly by polyamines. Expression of 2,742 genes was detected in cells cultured with or without putrescine. Among these, 309 genes were upregulated (more than twofold increase) and 319 genes were downregulated (less than half) by polyamines (Yoshida et al. 2004). Polyamines thus "modulate" the level of many kinds of proteins to maintain optimal conditions of cell growth.

We also studied the molecular mechanism of polyamine stimulation of protein synthesis. One of the examples is described here by showing polyamine effect on the synthesis of RMF, an important protein for cell viability (Terui et al. 2010). RMF is synthesized in the stationary phase and is uniquely associated with 100S

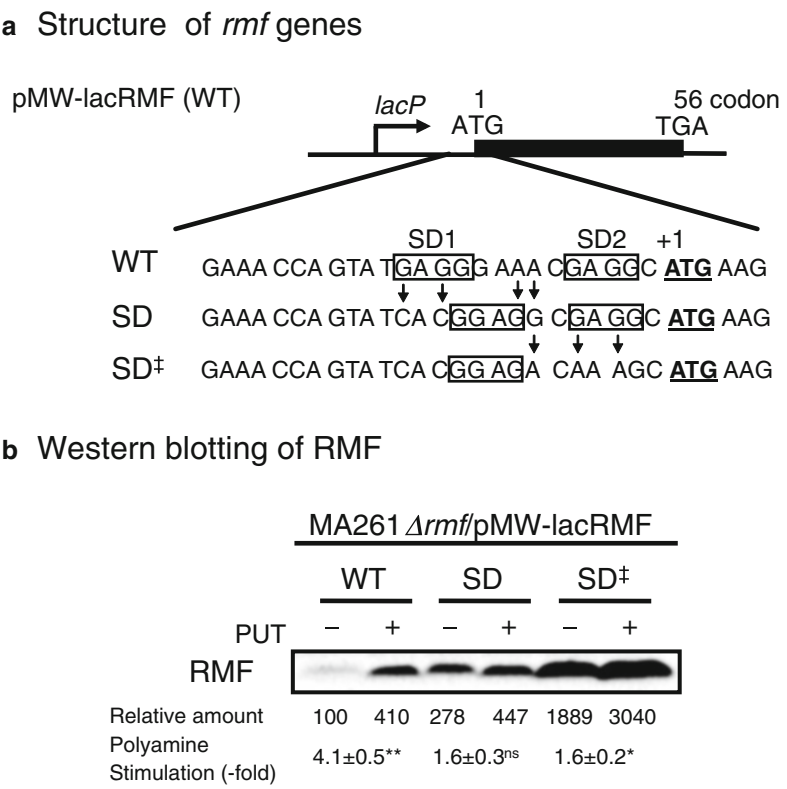


**Fig. 11.3** Effect of the position of Shine–Dalgarno (SD) sequence on polyamine stimulation of ribosome modulation factor (RMF) synthesis. **a** Nucleotide sequence of 5′-untranslated region of *rmf* mRNA. **b** *E. coli* MA261Δ*rmf* carrying pMW-lacRMF (wild type, WT), pMW-lacRMF (SD), or pMW-lacRMF(SD‡) was cultured in the presence and absence of 100 μg/ml putrescine. Western blotting of RMF synthesized from wild type and SD sequence modified *rmf* mRNAs was performed using 5 μg protein. Values are means ± standard errors of triplicate determinations. ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$

ribosome dimers (Wada et al. 1995). When the nucleotide sequence of *rmf* mRNA was determined, two candidate SD sequences were observed (Fig. 11.3a). Thus, two modified *rmf* mRNAs were constructed. In one construct, pMW-lacRMF(SD), the first SD sequence (SD1) located at 11 nucleotides upstream of the initiation codon AUG, was shifted to the more common position located at eight nucleotide upstream of the initiation codon. In the other construct, pMW-lacRMF(SD‡), the second SD sequence (SD2) was eliminated together with shifting of first SD1 sequence. When the *rmf(SD)* mRNA was used instead of *rmf(WT)* mRNA, the degree of polyamine stimulation was reduced from 4.1- to 1.6 fold, although the level of RMF protein in cells cultured without polyamines was increased. When the *rmf(SD‡)* mRNA was used, the degree of polyamine stimulation was also reduced from 4.1- to 1.6 fold,

and the level of RMF was markedly increased about 19 fold in cells cultured without polyamines because of the elimination of the inhibitory SD sequence located at the two nucleotides upstream of the initiation codon (Fig. 11.3b). The results indicate that synthesis of RMF is enhanced by polyamines because of the presence of an unusual SD sequence in *rmf* mRNA, as seen in other mRNAs.

We have reported that polyamine stimulation of OppA synthesis in *E. coli* and rat liver Ile-tRNA formation likely involves a structural change of the bulged-out region of double-stranded RNA (Igarashi and Kashiwagi 2010; Kusama-Eguchi et al. 1991) and have shown that a selective structural change of the bulged-out region of *oppA* mRNA and rat liver $tRNA^{Ile}$ is important for polyamine stimulation (Higashi et al. 2008). Thus, a possible secondary structure of the initiation region of *rmf* mRNA (−65 to +65) was constructed, and it was found that a bulged-out region existed in which selective structural change by spermidine would occur (Fig. 11.4a). The nucleotide sequence of the bulged-out region of double-stranded RNA, near the initiation codon AUG and the SD sequence of *rmf* mRNA, was modified to make three different forms of the double-stranded RNA. Those mRNAs are *rmf[−6(C → G)]* mRNA, *rmf[−35(ΔU)]* mRNA, and *rmf[−36(ΔC)]* mRNA. As a control, *rmf[+56(C → G)]* mRNA was constructed to remove the bulged-out region of another double-stranded region that is located distantly from the initiation site of *rmf* mRNA.

The effects of polyamines on the synthesis of an RMF-β-Gal fusion protein were studied using these various *rmf-lacZ* fusion mRNAs. As shown in Fig. 11.4b, polyamines stimulated the synthesis of RMF-β-Gal from wild-type *rmf-lacZ* mRNA by 4.6 fold. Polyamine stimulation of the synthesis of RMF-β-Gal fusion protein from *rmf[−6(C → G)]-lacZ*, *rmf[−35(ΔU)]-lacZ*, and *rmf[−36(ΔC)]-lacZ* mRNAs was reduced from 4.6 fold to 1.1- to 2.2 fold, although the basal level of protein synthesis in the absence of polyamines was enhanced. When polyamine stimulation of the synthesis of the RMF-β-Gal fusion protein from *rmf[+56(C → G)]-lacZ* mRNA was examined as a control, the degree of polyamine stimulation was nearly equal to that of synthesis from wild-type *rmf-lacZ* mRNA. These results support the idea that a structural change of the bulged-out region of double-stranded RNA close to the initiation codon AUG and the SD sequence of *rmf* mRNA is involved in polyamine stimulation of RMF synthesis. In parallel with the increase in RMF synthesis from the mutated *rmf* mRNAs, cell viability cultured without putrescine increased greatly (Terui et al. 2010). The results indicate that RMF is intensely involved in the increase in cell viability (see Fig. 11.2).

## 11.3 Polyamine Modulon in Eukaryotes

We next looked for protein(s) whose synthesis is stimulated by polyamines at the level of translation using a polyamine-requiring mutant of *Saccharomyces cerevisiae* deficient in ornithine decarboxylase (YPH499Δ*spe1*). When the YPH499Δ*spe1* mutant was cultured in polyamine-free medium, spermidine content was negligible (1.1 nmol/mg protein) and the rate of cell growth was delayed. When 0.1 mM

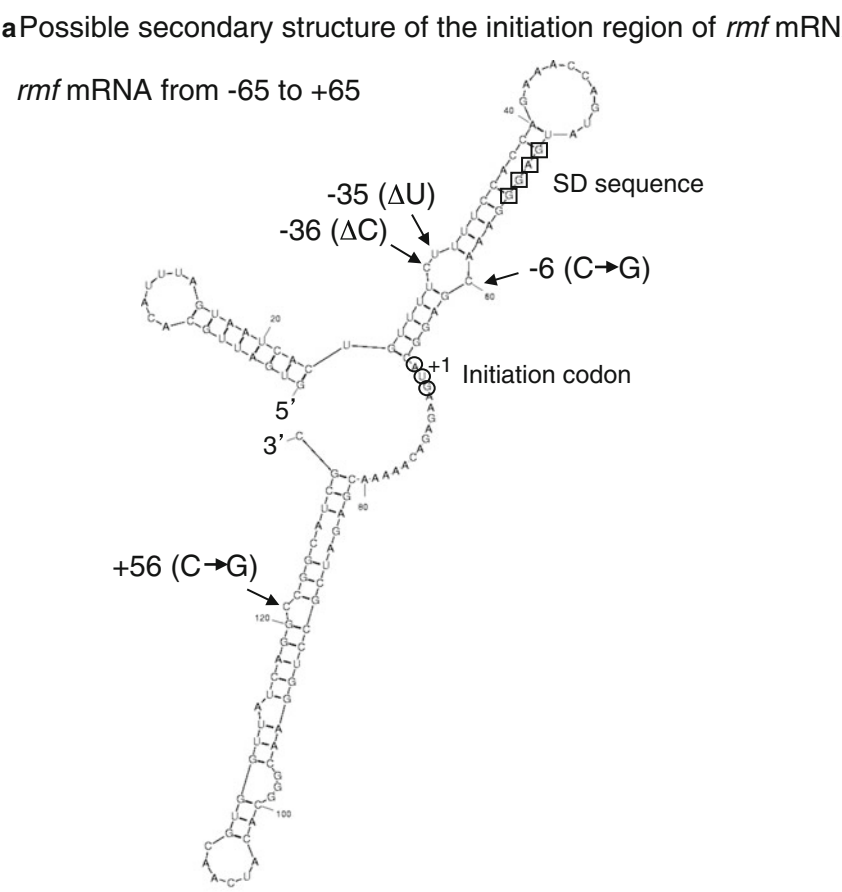


**Fig. 11.4** Effect of polyamines on the synthesis of RMF-β-Gal fusion proteins derived from wild and mutated *rmf-lacZ* mRNAs in the 5′-untranslated region. **a** Possible secondary structure of the initiation region of *rmf* mRNA showing the positions in which the sequence was mutated. Symbols of *filled triangles* and *right arrows* in *parentheses* represent removal and replacement of nucleotide, respectively. **b** Effect of polyamines on RMF-β-Gal fusion protein synthesis was evaluated by Western blot analysis. ns, $p > 0.05$; **, $p \leq 0.01$

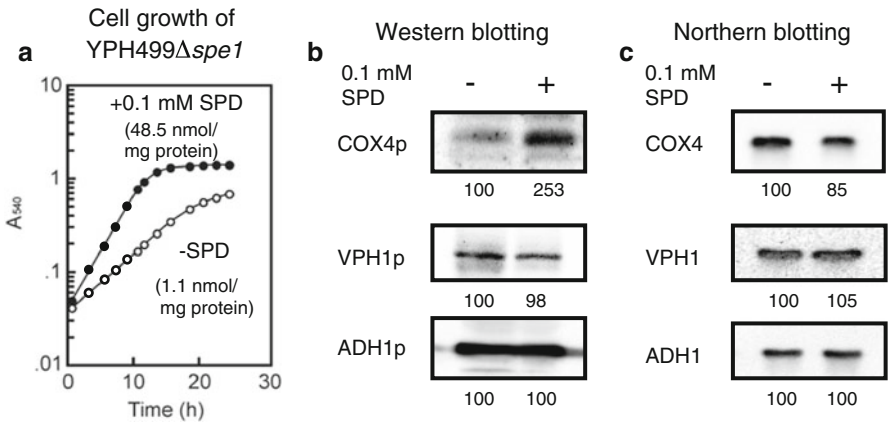


**Fig. 11.5** Effect of spermidine on cell growth of yeast polyamine-requiring mutant YPH499Δ*spe1*, and levels of protein and mRNA of COX4. **a** A polyamine-requiring mutant YPH499Δ*spe1* was cultured in the presence (*closed circles*) and absence (*open circles*) of 0.1 mM spermidine, and cell growth was followed by measuring $A_{540}$. **b**, **c** The levels of COX4 protein and mRNA, together with those of VPH1 and ADH1. The level in the presence of spermidine compared with that in the absence of spermidine is shown as percent. Values are means of three experiments

spermidine was added to the medium, spermidine content increased to 48.5 nmol/mg protein and the rate of cell growth was increased 2- to 3 fold (Fig. 11.5a). Under these conditions, synthesis of COX4, one of the subunits of cytochrome C oxidase (complex IV), was enhanced by polyamines about 2.5 fold at the level of translation (Fig. 11.5b, c) (Uemura et al. 2009). As a control, levels of vacuolar $H^+$-ATPase (VPH1) and aldehyde dehydrogenase (ADH1) were measured. These levels were similar in cells cultured with or without spermidine.

To study the mechanism of spermidine stimulation of COX4 synthesis, a fusion gene was constructed in a vector containing 122 nucleotides of the 5′-untranslated region (5′-UTR) and 105 nucleotides of the open reading frame (ORF) of the COX4 gene fused to the EGFP gene. It was found that the level of COX4-EGFP protein was higher in cells cultured with spermidine than in cells cultured without spermidine, whereas the level of COX4-EGFP mRNA was nearly equal with or without spermidine (Fig. 11.6). This finding was confirmed by fluorescence of EGFP, which was more intense in cells cultured with spermidine than in cells cultured without spermidine. The results strongly suggest that a structural change of the 5′-UTR of COX4 mRNA by spermidine is involved in the spermidine stimulation of COX4 synthesis. There are two stem-loop (hairpin) structures in the 5′-UTR of COX4 mRNA (Fig. 11.6a), and it has been reported that COX4 synthesis is regulated through the binding of a repressor protein to hairpins I and II (Su and Dowhan 2006). To determine whether spermidine enhances ribosome shunting, an initiation codon AUG was inserted in loop I and loop II to be out of frame with normal AUG (Fig. 11.6a). If initiation of protein synthesis occurs by scanning of the 40S ribosomal subunit from cap to the initiation codon AUG, only a short peptide could be

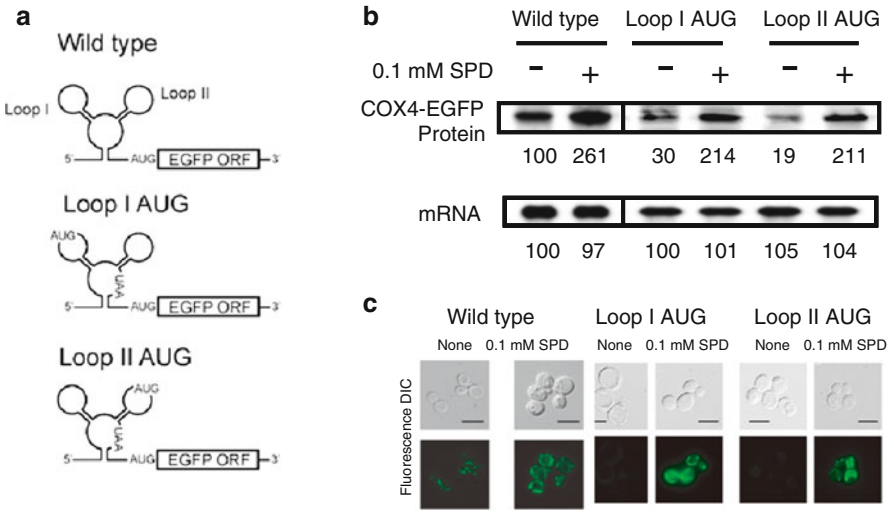


**Fig. 11.6** Effect of insertion of initiation codon AUG in loop I or II in the 5′-UTR of COX4-EGFP mRNA on COX4-EGFP synthesis. **a** Schematic structure of COX4-EGFP mRNA containing inserted initiation codon AUG in loop I and loop II. **b** The level of COX4-EGFP protein and mRNA expressed from YEpCOX4-EGFP, YEpCOX4 (loop I AUG)-EGFP and YEpCOX4 (loop II AUG)-EGFP was measured; the level in the presence of spermidine compared with that in the absence of spermidine is shown as *percent*. Values are means of three experiments. **c** Differential interference contrast (DIC, *top panels*) and corresponding fluorescence image of COX4-EGFP (*bottom panels*). *Bar* 10 μm

synthesized instead of COX4-EGFP fusion protein because a termination codon UAA for the inserted initiation codon AUGs exists in the 83th to 85th nucleotide of the 5′-UTR of mRNA (Fig. 11.6a). If ribosome shunting of hairpins I and II is enhanced by spermidine, a COX4-EGFP fusion protein could be synthesized because the inserted initiation codon AUGs are not recognized by 40S ribosomal subunits. Synthesis of COX4-EGFP in the absence of spermidine decreased greatly compared with that synthesized from intact COX4-EGFP mRNA (Fig. 11.6b,c). However, it was strongly enhanced by spermidine. The degree of spermidine stimulation was more than sevenfold when the initiation codon was inserted into the 5′-UTR of COX4-EGFP mRNA. Our results support the idea that a short peptide is preferentially synthesized by 5′-processive scanning of 40S ribosomal subunits in the absence of spermidine, whereas COX4-EGFP can be synthesized by ribosome shunting of the hairpin structures of 5′-UTR in the presence of spermidine.

We then looked for proteins whose synthesis is enhanced by polyamines at the level of translation using a mouse mammary carcinoma FM3A cell culture system (Nishimura et al. 2009). Polyamine deficiency was induced by adding an inhibitor of ornithine decarboxylase, α-difluoromethylornithine, to the medium. Proteins enhanced by polyamines were determined by comparison of protein levels in control and polyamine-deficient cells using two-dimensional gel electrophoresis and were identified by Edman degradation and/or LC/MALDI-TOF tandem mass

spectrometry. Polyamine stimulation of these proteins at the level of translation was confirmed by measuring levels of corresponding mRNAs and proteins and levels of the [$^{35}$S]methionine pulse-labeled proteins. The proteins identified in this way were T-complex protein 1, β-subunit (Cct2), heterogeneous nuclear riboprotein L (Hnrpl), and phosphoglycerate mutase 1 (Pgam1). Because Cct2 was most strongly enhanced by polyamines among the three proteins, the mechanism of polyamine stimulation of Cct2 synthesis was studied using NIH3T3 cells transiently transfected with genes encoding Cct2-EGFP fusion mRNA with normal or mutated 5′-UTR of Cct2 mRNA. Polyamines enhanced ribosome shunting on the 5′-UTR of Cct2 mRNA similar to COX4 synthesis in yeast.

## 11.4 Future Perspectives

Physiological functions of polyamines are gradually being clarified at the molecular level. Our results strongly suggest that polyamine effect on cell proliferation and cell viability mainly occurs at the level of translation. In *E. coli*, 17 kinds of protein synthesis are enhanced by polyamines at the level of translation. Furthermore, polyamines stimulate the assembly of 30S ribosomal subunits (Igarashi and Kashiwagi 2010). Thus, it is clear that polyamines contribute to cell proliferation and cell viability of *E. coli* mainly through interaction with RNA.

In eukaryotes, it was recently found that four kinds of genes are members of the polyamine modulon. However, this is not enough to explain polyamine effect on cell growth. We expect that more than 30 kinds of polyamine modulon exist in eukaryotes. In addition, eukaryotic initiation factor 5A (eIF5A) contains hypusine, which is a modified lysine with the addition of the 4-aminobutyl moiety from spermidine. It is known that eIF5A is also involved in protein synthesis (Park et al. 2010). We have shown that polyamines and eIF5A are independently involved in cell growth (Nishimura et al. 2005). These findings also support the idea that polyamines involved in cell proliferation and viability through regulation of protein synthesis.

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# Chapter 12
# Protein Synthesis and Polyamines in Thermophiles: Effect of Polyamines on Nucleic Acid Maintenance and Gene Expression

**Shinsuke Fujiwara, Ryota Hidese, Takahiro Inoue, and Wakao Fukuda**

**Abstract** Gene expression at both the transcriptional and translational levels is critically dependent upon DNA and RNA structure, particularly in hyperthermophiles, which grow at temperatures above 80 °C. Nucleosome-like structures (histone-bound DNA) from hyperthermophilic Archaea are compacted and stabilized in the presence of multivalent polyamines, suggesting that polyamines play a role in nucleosome maintenance in hyperthermophiles. Multivalent polyamines inhibit the melting of double-stranded DNA and structured RNA. Longer-chain polyamines stabilize double-stranded nucleic acids, whereas branched-chain polyamines stabilize stem-and-loop structures, suggesting that branched-chain polyamines are involved in gene translation. Protein synthesis catalyzed by a cell-free extract of the hyperthermophilic archaeon, *Thermococcus kodakarensis*, requires the presence of longer- and/or branched-chain polyamines. Translational activity increases in the presence of a variety of linear polyamines and is dependent on chain length. Putrescine and spermidine do not increase translational activity. By contrast, longer polyamines such as homocaldopentamine [3334], caldopentamine [3333], and thermopentamine [3343] increase translational activity. The greatest activity

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occurs in the presence of $N^4$-bis(aminopropyl)spermidine [3(3)(3)4] (abbreviation for the number of methylene $CH_2$ chain units between $NH_2$, NH, N, or $N^+$). In vitro experiments using cell extracts from the thermophilic bacterium, *Thermus thermophiles*, reveal that branched-chain polyamines appear to play a role in peptide bond formation during protein biosynthesis. Thus, it appears that branched-chain polyamines are essential for the proper formation of the 30S initiation complex, which acts as the initial aminoacyl-tRNA in thermophiles.

**Keywords** Archaea • Branched-chain polyamine • Cell-free translation • Nucleosome • Thermophile

## 12.1 Introduction

In thermophiles, DNA- or RNA-binding substances are thought to be involved in thermal protection. Polyamines are positively charged under physiological conditions; therefore, they bind electrostatically to the negatively charged phosphate groups within DNA and RNA, thereby protecting these molecules from thermal denaturation (Morgan et al. 1987; Tabor and Tabor 1984). This interaction also has a significant effect on gene expression. Here, we describe the role of polyamines in the maintenance of nucleosome structure and gene expression.

## 12.2 Stabilization of Nucleic Acids by Unique Polyamines

Thermophiles (both bacterial and archaeal) produce two types of unusual polyamine: long linear polyamines (e.g., caldopentamine [3333] and caldohexamine [33333]), and branched polyamines (e.g., tris-(3-aminopropyl)amine [3(3)3], $N^4$-aminopropylspermidine [3(3)4], tetrakis-(3-aminopropyl)ammonium [3(3)(3)3], and $N^4$-bis(aminopropyl) spermidine [3(3)(3)4]) (Fig. 12.1) (Oshima and Kawahata 1983; Hamana et al. 1992, 1994). These polyamines are believed to have a role in thermal protection.

In the absence of polyamines, the stability of nucleic acids is dependent on structural features. For example, the DNA double helix is stabilized primarily by two forces: hydrogen bonds between nucleotides, and base-stacking interactions between aromatic nucleobases. Some combinations of base pairs form more stable interactions than others, so nearest neighbor base-stacking interactions are important determinants of duplex stability. Base-stacking interactions increase with increasing salt concentration, as high salt concentrations mask the (destabilizing) charge repulsion between the two negatively charged phosphodiester backbones. Divalent cations such as $Mg^{2+}$ are more stabilizing than monovalent cations such as sodium ($Na^+$) and potassium ($K^+$), and some metal ions bind to specific loci on the DNA duplex. Several species of hyperthermophiles possess high cytoplasmic concentrations of potassium ions. For example, the cytoplasmic potassium concentrations of *Methanothermus fervidus* and *Pyrococcus woesei* are approximately 1.0 and 0.6 M, respectively

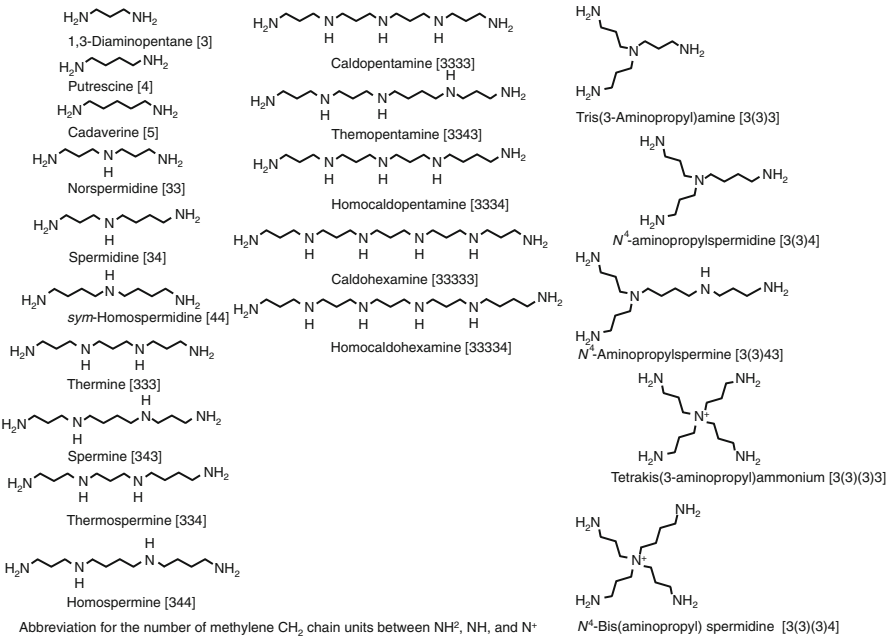


**Fig. 12.1** Structure of linear and branched polyamines

(Hensel and Konig 1988; Scholz et al. 1992). Finally, high intracellular salt concentrations also appear to prevent DNA strand separation in vivo (Bowater et al. 1994). Besides potassium ions, archaeal histones, which are thought to have evolved from a common ancestor and are likely to have structures and functions similar to those of eukaryotic histones, bind to and wrap around the DNA strand, contributing to compaction (Sandman et al. 1990; Maruyama et al. 2011). Histones and polyamines act synergistically to stabilize double-stranded DNA against thermal denaturation. The effect of polyamines on histone-induced nucleosomes has been investigated using archaeal histones (HpkA and HpkB) from *Thermococcus kodakarensis* (Higashibata et al. 2000). DNA bound to a polyamine (putrescine [3], spermidine [34], or spermine [343]) showed the same degree of compaction as naked DNA; however, HpkA-DNA complexed with a polyamine showed a higher degree of compaction than HpkA-DNA in the absence of the polyamine, indicating that polyamines induce a more compacted form. Compaction is enhanced as the valence number of the polyamine increases. Putrescine is a divalent polyamine that possesses a "$2^+$" charge at physiological pH, whereas spermidine ($3^+$) and spermine ($4^+$) are trivalent and tetravalent, respectively. Spermine is the most effective in terms of inducing compaction. The spermine molecule crosses the major groove of the DNA strand, and the nitrogen atoms within the central part of the molecule form hydrogen bonds with the –OH or –NH groups of the nucleic acid bases (Drew and Dickerson 1981). HpkA-DNA shows identical levels of compaction when complexed with divalent compounds, with $N^1$-acetylspermidine and $N^8$-acetylspermidine (which also possess a "$2^+$" charge), or with trivalent compounds such as spermidine and $N^1$-acetylspermine.

The acetylated form of a polyamine is generally less effective for DNA compaction, possibly because of reduction in binding affinity resulting from the reduced electrostatic valence. Double-stranded DNA is stabilized by high cytoplasmic concentrations of potassium chloride, whereas compacted DNA is stabilized by polyamines and acetylpolyamines. It is noteworthy that polyamines are generally more effective at stabilizing compacted DNA than acetylpolyamines. We recently identified acetylated polyamines in *T. kodakarensis*, suggesting that acetylation and deacetylation play roles in gene regulation by altering nucleosome structure (unpublished data). Thermal denaturation of HpkA-DNA complexed with spermine or potassium chloride was examined by measuring the absorbance (260 nm) of double-stranded poly(dA-dT), which is thought to be the most thermolabile double-stranded DNA (Fig. 12.2). The results revealed that poly(dA-dT) showed a melting temperature of 47.5 °C, which shifted to 63.5 °C and 65.5 °C in the presence of HpkA and spermine, respectively. When both HpkA and spermine were added, the melting point shifted to 75.0 °C. The melting point of double-stranded poly(dA-dT) in the presence of potassium chloride was >76.5 °C; however, no further increases were observed upon addition of HpkA. These data suggest that high cytoplasmic concentrations of potassium chloride stabilize double-stranded DNA, and that polyamines stabilize compacted DNA. Hou et al. examined how $Na^+$, $Mg^{2+}$, and polyamines (spermidine and spermine) affected the stability of various DNA oligonucleotides containing sequences that mimic various cellular DNA structures, including duplexes, bulged loops, hairpins, and/or mismatched base pairs (Hou et al. 2001). Melting temperature

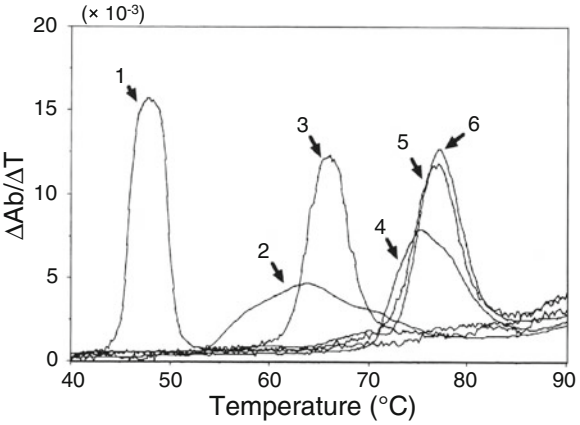


**Fig. 12.2** Melting profile of poly(dA-dT) DNA. poly(dA-dT) double-stranded DNA (10.0 μg) was mixed with HpkA (17.3 μg; final concentration, 2.34 μM) in the presence or absence of spermine or potassium chloride, and thermal denaturation of the complex was examined according to the method of Higashibata et al. (2000). ΔAb/ΔT was plotted against temperature for six different mixtures: *1*, poly(dA-dT) alone; *2*, poly(dA-dT) plus HpkA (2.34 μM); *3*, poly(dA-dT) plus spermine (10 μM); *4*, poly(dA-dT) plus HpkA (2.34 μM) and spermine (10 μM); *5*, poly(dA-dT) plus potassium chloride (1.0 M); and *6*, poly(dA-dT) plus HpkA (2.34 μM) and potassium chloride (1.0 M). The calculated melting temperatures for mixtures 1 to 6 were 47.5, 63.5, 65.5, 75.0, 76.5, and 77.0 °C, respectively

curves indicated that these cations stabilized duplex formation in the order spermine > spermidine > $Mg^{2+}$ > $Na^{+}$ > Tris-Cl buffer alone (at pH 7.3). Another study compared the effects of unique long-chain or branched-chain polyamines with those of standard polyamines (Terui et al. 2005; Oshima 2007). When the stability of synthetic DNA was examined in the presence of a variety of linear polyamines, the melting temperature ($T_m$) increased with the length of the polyamine. Moreover, the $T_m$ was higher in the presence of homocaldopentamine [3334] (which contains one aminobutyl group) than in the presence of caldopentamine [3333] (which comprises only aminopropyl groups). Longer polyamines may effectively stabilize double-stranded structures, such as DNA and the stem portions of RNA, in thermophile cells at physiological temperatures. Aminobutyl-containing polyamines are more effective at stabilizing double-stranded structures than polyamines comprising only aminopropyl groups (e.g., caldopentamine and caldohexamine) (Oshima 2007). Also, long polyamines suppress the depurination of DNA at high temperatures (Oshima 2007). Branched polyamines do not obey the empirical rules just detailed; the $T_m$ in the presence of a branched polyamine was lower than that in the presence of its isomeric linear polyamine. For example, the $T_m$ of a synthetic DNA oligomer in the presence of tetrakis(3-aminopropyl)ammonium [3(3)(3)3] was 2.3 °C lower than that in the presence of caldopentamine [3333], although these two polyamines are structural isomers (their chemical formulae are identical, $C_{12}H_{36}N_5$) and they comprise four aminopropyl groups. When the stability of a purified tRNA was examined in the presence of several polyamines, the highest $T_m$ was recorded in the presence of tetrakis(3-aminopropyl)ammonium [3(3)(3)3]. It is thought that long polyamines and branched polyamines share the responsibility for stabilizing nucleic acids in thermophile cells; long polyamines stabilize double-stranded DNA and the stem structure of RNAs, and branched polyamines stabilize the loop structure of RNAs (Oshima 2007). Tetrakis(3-aminopropyl)ammonium [3(3)(3)3] stabilizes the RNA kissing dimer that is involved in the dimerization initiation site within HIV-1 genomic RNA. The metastable kissing dimer comprises a nine-nucleotide loop and a six-nucleotide self-complementary sequence, which is converted into a stable extended-duplex dimer (Imai et al. 2009). These findings suggest that branched polyamines function to protect RNA not only in thermophilic microorganisms but also in mesophilic ones.

## 12.3 Effect of Polyamines on Translation Efficiency

Cell-free expression experiments show that polyamines play several roles. In *Escherichia coli*, the synthesis of oligopeptide binding protein (OppA) (which is important for the supply of nutrients), adenylate cyclase (Cya), the RNA polymerase $\sigma^{38}$ subunit (RpoS), the transcription factor of iron transport operon (FecI), and transcription factors for growth-related genes (including rRNA and some kinds of tRNA, e.g., Fis) is enhanced by polyamines at the translational level. The group of genes whose expression is enhanced by polyamines at the level of translation is referred as the "polyamine modulon" (Igarashi and Kashiwagi 2006).

In thermophiles, long- and/or branched-chain polyamines appear to be involved in gene translation and are particularly effective at increasing translational efficiency at high temperatures. Different thermophiles synthesize different long- and branched-chain polyamines. For example, *Pyrobaculum aerophilum* and *Hyperthermus butylicus* synthesize long-chain polyamines (Knott 2009). On the other hand, *T. kodakarensis* has no linear long polyamines but does express $N^4$-bis(aminopropyl) spermidine [3(3)(3)4] and some $N^4$-aminopropyl spermidine [3(3)4] (both branched-chain polyamines) (Morimoto et al. 2010; Okada et al. 2014). Appropriate long and/or branched polyamines appear to be synthesized in response to the physiological temperature in the natural environment: branched-chain polyamines are abundant in thermophiles grown at high temperatures (such as 80 °C), but form only a minor component of the polyamine pool in cells grown at relatively lower temperatures (Morimoto et al. 2010; Oshima et al. 1987). Crude cell-free extracts (specifically the S30 fraction) are useful tools for investigating biochemical phenomena and for exploiting complex enzymatic processes such as protein synthesis. The S30 fraction has been used to investigate the role of polyamines in hyperthermophiles. As already mentioned, it is thought that long polyamines and branched polyamines share the responsibility for stabilizing nucleic acids (Terui et al. 2005; Oshima 2007). The diameter of the tRNA loop is 13 Å, which is almost identical to the length of a spermine molecule [343]. We examined the effect of polyamines on translation using cell-free extracts obtained from *T. kodakarensis* grown at 60 °C. *T. kodakarensis* expressed higher levels of $N^4$-bis(aminopropyl)spermidine [3(3)(3)4] when the cells were cultivated at temperatures >85 °C, whereas lower levels were synthesized at 60 °C (Morimoto et al. 2010; Okada et al. 2014). As shown in Fig. 12.3, relative translational activity in the presence of a variety of linear polyamines increases with chain length. Putrescine and spermidine do not induce a significant increase in translational activity, whereas longer polyamines such as homocaldopentamine [3334], caldopentamine [3333], and thermopentamine [3343] do increase translational activity. It is worth noting that caldohexamine [33333] does not increase activity. In larger polyamines with a valency greater than 2 (e.g., spermidine), the carbon chain bends at the position of nitrogen attachment. For example, in the case of thermopentamine [3334], the polyamine molecule can be stably packed by bending within the tRNA loop. By contrast, caldohexamine [33333] exceeds the packaging limit, even though the molecule is able to bend (as shown in Fig. 12.3c). The relative translational activity in the presence of the quaternary polyamines, $N^4$-bis(aminopropyl)spermidine [3(3)(3)4] and $N^4$-aminobutylaminopropylspermidine [3(3)(4)4], is higher than that in the presence of tertiary polyamines (Fig. 12.4). Quaternary polyamines, including tetrakis-(3-aminopropyl)ammonium [3(3)(3)3], might possess the most suitable structure for packaging within the loop region of tRNA. Putrescine and spermidine have a negative effect on translational activity (Fig. 12.3), because small polyamines might be replaced with preexisting branched polyamines in the cytoplasm, such as $N^4$-bis(aminopropyl)spermidine [3(3)(3)4] derived from *T. kodakarensis* S30, which might inhibit translational activity. Kawai et al. investigated the interaction between tetrakis(3-aminopropyl)ammonium [3(3)(3)3] and yeast phenylalanyl-tRNA using

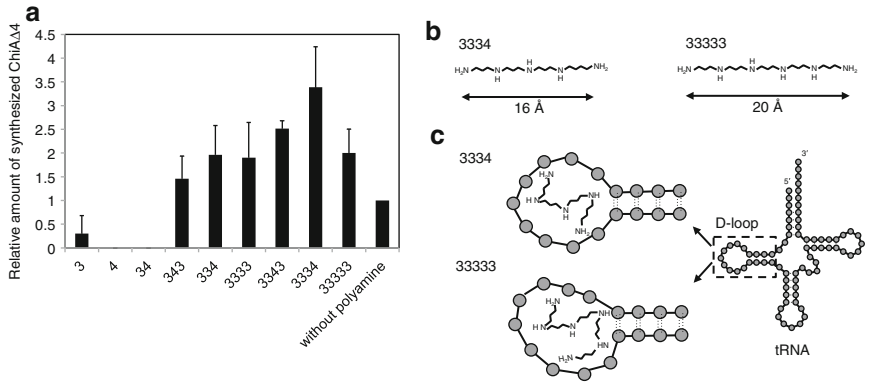


**Fig. 12.3** Effect of linear polyamines on translational activity. **a** Relative translation activity in the presence or absence of linear polyamines examined in a cell-free protein synthesis system incorporating the S30 fraction from *T. kodakarensis* KC1 strain (which lacks the gene for ChiAΔ4, a derivative of chitinase). Reaction mixtures containing 0.4 mg/ml of ChiAΔ4 mRNA were incubated at 80 °C for 90 min in the presence of 0.2 mM of each linear polyamine. ChiAΔ4 synthesis was measured in a chitinase activity assay (Endoh et al. 2007). As a negative control, the reaction was performed in the absence of polyamine. The cell-free protein synthesis levels were normalized to that of the negative control, which was set to 1. Results are expressed as the mean ± SD of three independent reactions. **b** Structure and length of homocaldopentamine [3334] and caldohexamine [33333]. **c** The speculated binding mode between linear polyamines and tRNA loop regions. *Circles* indicate nucleotide unit

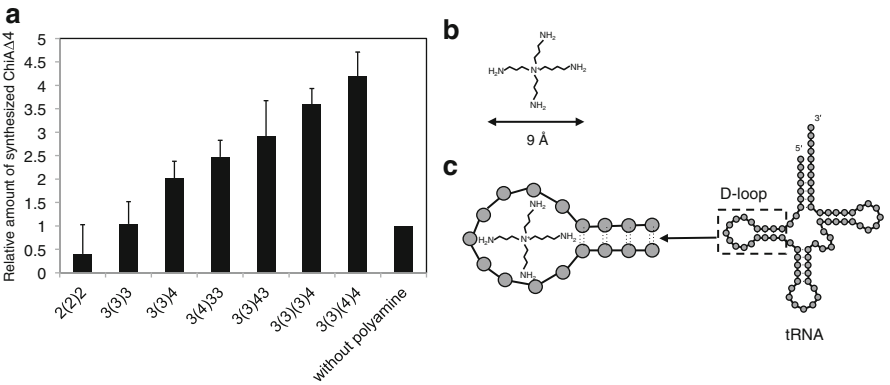


**Fig. 12.4** Effect of branched polyamines on translational activity. **a** Relative translation activity in the presence or absence of linear polyamines was examined in a cell-free protein synthesis system incorporating extracts from *T. kodakarensis* KC1 (which lacks the gene for ChiAΔ4, a derivative of chitinase) at 80 °C for 90 min. Each branched polyamine was added to a reaction mixture. The relative amounts of synthesized ChiAΔ4 were measured as described in Fig. 12.3. **b** Structure and chain length of $N^4$-bis(aminopropyl)spermidine. **c** The speculated binding mode between branched-chain polyamines and tRNA loop regions. *Circles* indicate nucleotide unit

nuclear magnetic resonance (unpublished data). The preliminary results suggest that the branched polyamine interacts with nucleotide residues within the D-loop of the tRNA (Oshima 2007) and may induce a conformational change in the tRNA. A precise in vitro study using cell-free extracts from *T. thermophilus* showed that protein synthesis at high temperatures (65 °C or higher) requires polyamines for efficient translation (Ono-Iwashita et al. 1975). Reactions at lower temperatures (e.g., 37 °C) did not require polyamines, although activity was poor. No activity was detected at 65 °C or above in the absence of polyamines, as their complete removal (by dialysis) inactivated the ribosomes. The addition of linear polyamines, such as tetraamine, or branched polyamines, such as tetrakis(3-aminoproppyl)ammonium [3(3)(3)3], rescued cell-free protein synthesis. It is thought that polyamines do not prevent the thermal denaturation of protein(s) or nucleic acid(s) involved in protein biosynthesis, because full activity was observed in an experiment in which a polyamine was added after 5 min of incubation at 65 °C (Oshima 2007). Uzawa et al. examined the effects of linear polyamines (comprising only aminopropyl groups) on cell-free protein synthesis, and found that thermine [333] > caldopentamine [3333] > caldohexamine [33333] >> norspermidine [33]; that is, the tetraamine had the greatest effect (Uzawa et al. 1993). In addition, polyamines containing an aminobutyl group increase the translation reaction rate more effectively than homologues containing only aminopropyl groups. For example, spermine [343] and thermospermine [334] show higher enhancing effect than thermine [333]. Although aminopropyl and aminobutyl groups have different effects on translation, there are no differences in their effects on DNA stability. The effects of polyamines on DNA conformation appear to be related to the number of positive charges that the polyamine possesses (Wilson and Bloomfield 1979).

It is thought that polyamines are essential for the formation of the initiation complex, which comprises the 30S ribosomal subunit, the mRNA, and the initial aminoacyl-tRNA. When poly(U) was used as a mRNA, the highest level of activity was recorded in the presence of tetrakis(3-aminopropyl)ammonium (Uzawa et al. 1993). This conclusion was obtained when peptide bond formation was measured using phenylalanyl-tRNA as the substrate for the reaction. Polyamines are thought to be involved in protein biosynthesis. The addition of a tetraamine (e.g., spermine) to a cell-free protein synthesis mixture containing tetrakis(3-aminoproppyl) ammonium led to a synergistic increase in activity only at temperatures >65 °C (Uzawa et al. 1993). When a thermophile extract was used as the catalyst, only tetrakis(3-aminoproppyl)ammonium inhibited phenylalanyl-tRNA formation to a significant extent, and the inhibition was rescued by the addition of other polyamines (Uzawa et al. 1994). The formation of glycyl-tRNA was moderately inhibited by two branched polyamines [tetrakis(3-aminoproppyl)ammonium and tris(3-aminopropyl)amine], and that of isoleucyl-tRNA was moderately inhibited by a quaternary amine; however, the inhibition was not as significant as that observed for phenylalanyl-tRNA. Polyamines also enhanced the elongation step, although they were not essential. Similar observations were made in a study examining in vitro protein synthesis using a *Sulfolobus acidocaldarius* cell-free extract and poly(U) (Friedman and Oshima 1989).

## 12.4 Conclusion

Long polyamines and branched polyamines stabilize nucleic acids in thermophiles; long polyamines stabilize double-stranded DNA and the RNA stems, whereas branched polyamines stabilize the RNA loops. Both are involved in protein biosynthesis and play a significant role in translation at high temperatures. Polyamines are involved in a wide variety of cellular regulatory pathways in eukaryotic cells, including those that control the equilibrium between cellular proliferation and apoptosis (Lindsay and Wallace 1999) and the induction of cellular differentiation (Wallace et al. 2003). Polyamines also fulfill unknown functions in thermophiles, because mutant strains lacking the ability to synthesize long branched polyamines show significantly reduced cell growth and loss of thermotolerance (Morimoto et al. 2010). Further studies are required to clarify the specific role(s) of unique polyamines in the translational machinery of thermophile cells.

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# Part V
# Roles of Polyamines in Survival

# Chapter 13
# Polyamines in Plant Stress Response

**Thomas Berberich, G.H.M. Sagor, and Tomonobu Kusano**

**Abstract** Polyamines (PAs) are ubiquitous biogenic amines that have been implicated in diverse cellular functions in all organisms. Recent work has demonstrated that mainly the three prominent PAs in plants, putrescine, spermidine, and spermine, have a role as part of the complex signaling networks activated in response to abiotic stresses and pathogen attack. In a network comprising the PAs itself, their catabolic products such as $H_2O_2$ produced by action of diamine oxidases (DAO) and polyamine oxidases (PAO), and other compounds such as γ-aminobutyric acid (GABA), abscisic acid (ABA), and nitric oxide (NO), are part of interlaced cellular pathways. Current knowledge about PAs in plant stress responses is summarized in this chapter.

**Keywords** Cold • Drought • Heat • Pathogen • Polyamine • Salinity • Stress • Temperature • Tolerance

## 13.1 Introduction

As sessile organisms, plants are exposed to all imaginable environmental stresses and therefore have evolved diverse strategies to combat various life-threatening situations. Abiotic stresses include high and low temperature, water shortage and flooding, high and low radiation, and also salt and heavy metal stress. Biotic stresses range from viral, bacterial, and fungal infection to predators from insects to mammals. Besides morphological adaptations to severe habitats, different physiological and cellular pathways in stress response have evolved in plants and were identified. Plants can acquire tolerance to certain stresses by acclimatization during less severe

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stress periods, for example, freezing tolerance acquired at low temperatures above 0 °C. Modulation of polyamine levels in response to stress has been observed in different plant species (Bouchereau et al. 1999; Walters 2003a; Groppa and Benavides 2008; Kusano et al. 2008; Alcázar et al. 2010a), often accompanied by changes in expression levels of genes involved in polyamine biosynthesis (Alcázar et al. 2010b). From most of the studies it can be concluded that PA accumulation represents a stress-induced response with a protective role. Transgenic approaches in which polyamine biosynthetic genes have been overexpressed in plants support these observations. Extensive reviews on plant stress responses and also on the involvement of PAs have been published (Shinozaki et al. 2003; Gill and Tuteja 2010; Takahashi and Kakehi 2010; Krasensky and Jonak 2012; Alcázar et al. 2011a, b; Gupta et al. 2013). In this chapter, current knowledge of the involvement of polyamines in defense mechanisms against environmental stresses in plants is summarized.

## 13.2 High- and Low-Temperature Stress

Land plants need to differentiate between harmless and harmful changes in temperature to induce protective measures against damage and therefore have molecular thermometers to precisely sense such changes (Hua 2009; Mittler et al. 2012). In high temperatures, the so-called heat-shock response (HSR) leads to the accumulation of protective heat-shock proteins (HSPs) to overcome the period of harmful temperature (Kotak et al. 2007; Scharf et al. 2012; Qu et al. 2013). The expression of PA biosynthetic pathway genes upon heat shock was investigated in *Arabidopsis* (Sagor et al. 2012a). In that study, *SPMS* and *SAMDC2* genes were found to be induced in the early stage of the stress, followed by the *ADC2* gene. Correspondingly, the levels of Spm, Put, and Spd increased upon heat stress although that of T-Spm did not. Using transgenic *Arabidopsis* overexpressing *SPMS* and a Spm-deficient mutant plant, they found that the higher the endogenous Spm concentration the higher the thermotolerance (Sagor et al. 2012a). Thermotolerant tomato plants could be created by overexpressing a yeast *SAMDC* gene, that caused Spd and Spm levels to increase by 1.7- and 2.4 fold, respectively (Cheng et al. 2009). Enhanced antioxidant enzyme activities and protection of membrane lipid peroxidation as observed in these transgenic tomato plants were presumed to cause enhanced thermotolerance (Cheng et al. 2009). The PA metabolism under heat stress was also investigated in proline-overproducing tobacco plants in which a modified proline biosynthetic enzyme, Δ1-pyrroline-5-carboxylate synthetase, was overexpressed (Cvikrova et al. 2012). Corresponding to enhanced activities of the biosynthetic enzymes, the levels of Put, Spd, norspermidine (N-Spd), and Spm rose transiently after a 2-h lag phase. From the observations described here, an involvement of PAs in heat stress and thermotolerance can be hypothesized, but more data are necessary to prove such a mechanism.

Plant responses to low temperatures have been extensively investigated on gene transcription, protein, and also metabolic levels, mainly in the model plant *Arabidopsis thaliana* (Cook et al. 2004; Hannah et al. 2005; Kaplan et al. 2007; Usadel et al. 2008; Knight and Knight 2012; Barrero-Gil and Salinas 2013). For the role of PAs in low-temperature stress several data were accumulated (Cook et al. 2004; Alcázar et al. 2006, 2011b; Usadel et al. 2008). It was found in many plant species that under cold stress PA levels increase, especially in cold-tolerant lines. In wheat and alfalfa the Put levels increased during the process of cold-hardening (Nadeau et al. 1987) as well as in rice (Lee et al. 1997) and in bean (Guye et al. 1986). Further evidence for the protective role of Put comes from experiments in which exogenous Put could rescue tomato cold-induced electrolyte leakage while an inhibitor of ornithine decarboxylase increased the leakage (Kim et al. 2002). When *Arabidopsis* plants are exposed to low temperature, free Put accumulates after 24 h although Spd levels do not change. Associated with the change in Put level is the enhanced expression of the PA biosynthesis genes *ADC1* and *ADC2* after 30 min of cold exposure with *ADC1* being more prominent (Cuevas et al. 2008). In fact, the promoter region of the *ADC1* gene contains a CRT/DRE (C-repeat/dehydration-responsive element) that is the target of CBF/DREB1 (C-repeat binding factor; dehydration-responsive element-binding protein) transcription factors (TFs) of the APETALA2/ETHYLENE RESPONSE FACTOR family of *Arabidopsis* TFs (Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al. 1997; Liu et al. 1998). The promoter of *ADC2* contains five ABA-responsive elements (ABRE) that are responsible for the enhanced expression of the gene mediated by the de novo biosynthesis of the phytohormone ABA, which is a player in the drought stress response (Lang et al. 1994; Alcázar et al. 2006). In their work Cuevas et al. (2008) reported that there is no significant increase of free Spd and Spm after cold treatment, although the expression of *SAMDC2* is enhanced and its promoter contains one CRT/DRE, five ABRE, and four LTREs (low-temperature responsive element) (Alcázar et al. 2006). As outlined here and further illustrated by Alcázar et al. (2011b), the increase in Put under cold stress is a result of enhanced activity of ADC1 and ADC2. Vogel et al. (2005) described the participation of a cold-inducible C2H2 zinc finger-type transcription factor, *Zat12*, in this effect. *Zat12* expression not only increases in response to cold but also in oxidative stress condition (Rizhsky et al. 2004). As proposed, upstream of *Zat12* $Ca^{2+}$ influx, followed by phosphorylation/dephosphorylation events, leads to the activation of this transcription factor. The resulting increase in Put via activation of zeaxanthin leads to accumulation of ABA. Then, stress-related genes comprising ABREs in their promoter are induced to accomplish the metabolic changes necessary for survival during the stresses (Cuevas et al. 2009; Alcázar et al. 2011a; Gupta et al. 2013). Cold stress especially leads to damage in plants when the photosynthetic apparatus in leaves is exposed to light which then produces hazardous reactive oxygen species (ROS) (Crosatti et al. 2013). The function of PAs related to chloroplasts was investigated, and thylakoid-associated PAs have been found under diverse abiotic stress situations (Sfichi et al. 2004; Navakoudis et al. 2003, 2007; Demetriou et al. 2007). Exogenous treatment

of cucumber with Spd improved chilling tolerance of its photosynthetic apparatus with enhanced SAMDC enzyme activity in the more tolerant variety (He et al. 2002b). When Spd accumulation in chilled leaves was blocked by chemical inhibition of SAMDC, the chilling-induced damage was enhanced (Shen et al. 2000; He et al. 2002a). It was shown that Put can increase light energy utilization through stimulation of ATP synthesis in photophosphorylation, although Spd and Spm were less efficient (Ioannidis et al. 2006). However, the same group further showed that Spd and Spm are efficient stimulators of nonphotochemical quenching and at high concentrations are efficient uncouplers of photophosphorylation (Ioannidis and Kotzabasis 2007). The changes in the photosynthetic electron transfer rate through PAs lead to inactivation of active reaction centers and increase of dissipated energy (Ioannidis and Kotzabasis 2007).

The accumulated data on how PAs interact with the temperature stress responses are useful to further understand why many plants are able to develop thermotolerance while others cannot.

## 13.3 Drought and Osmotic Stress

Under water-limiting conditions, endogenous PA levels in plants increase and the ratio of the different PAs varies (Turner and Stewart 1986; Galston et al. 1997; Yang et al. 2007; Groppa and Benavides 2008; Do et al. 2013). Recently Do et al. (2013) compared the contents of PAs and selected metabolites directly related to PA metabolism in 21 rice cultivars under long-term drought stress. Furthermore, they analyzed the expression of genes encoding enzymes involved in the PA biosynthesis pathway. Under control conditions, free Put was the predominant PA followed by free Spd and Spm. Under drought stress Put levels decreased and Spm became the most prominent PA. Gene expression analysis revealed that ADC-dependent PA biosynthesis responds much more strongly to the stress than the ODC pathway (Do et al. 2013). These data support earlier observations in rice (Turner and Stewart 1986). Transgenic rice plants overexpressing the *ADC* gene of *Datura stramonium* showed higher drought tolerance resulting from the conversion of Put to Spd and Spm (Capell et al. 2004). In other species, Spm also seems to play a major role in drought stress. An *Arabidopsis* mutant that is unable to produce Spm was hypersensitive to drought and was rescued by exogenously applied Spm but not by Put or Spd (Yamaguchi et al. 2007). By combining their results of gene expression analysis and changes in PA levels, Do et al. (2013) suggest that during drought stress a pathway leads to the conversion of Put and Spd into the accumulation of Spm. This path was also proposed by Alcázar et al. (2011a) in *Arabidopsis* and the resurrection plant *Craterostigma plantagineum*. Liu et al. (2004) suggested that free Spd, free Spm, and bound Put facilitated the osmotic stress tolerance of wheat seedlings because PEG 6000 treatment significantly increased the free Spd and free Spm levels in leaves of a drought-tolerant variety whereas a drought-sensitive variety showed a significant increase of free Put level. Thus, the drought-tolerant variety showed a

higher ratio of (free Spd + free Spm)/free Put than the sensitive variety in response to osmotic stress. On the other hand, in many species Put accumulated in response to osmotic stress (Flores and Galston 1982; Galiba et al. 1993; Aziz and Larher 1995; Legocka and Kluk 2005), but these data are not as comprehensive as for the roles of Spm and/or Spd in drought stress response. It was shown for soybean pod and seeds that Spm is able to improve the defense against osmotic stress through antioxidants and abscisic acid changes (Radhakrishnan and Lee 2013). Exogenously applied Spm reduced lipid peroxidation, elevated the total content of polyphenol, and increased activities of antioxidant enzymes such as catalase and superoxide dismutase. Furthermore, synthesis of the plant hormone abscisic acid (ABA) was inhibited in osmotic stress pods after Spm treatment (Radhakrishnan and Lee 2013). ABA plays a pivotal role in a variety of stress responses especially in drought and osmotic stress (Seki et al. 2007; Fujita et al. 2011). As already described under temperature stress, the ADC2 gene contains five ABA-responsive elements (ABRE) in its promoter that are responsible for the enhanced expression of the gene mediated by ABA (Lang et al. 1994; Alcázar et al. 2006). *Arabidopsis* plants became more drought tolerant by overexpression of *ADC2*, which leads to higher Put levels and induction of stomata closure (Alcázar et al. 2010a). Supportive to this observation, ABA-deficient and ABA-insensitive mutants showed impaired Put accumulation in response to drought (Alcázar et al. 2006). The same authors described that overexpression of *SAMDC* leads to increased Spm, which in turn enhanced the induction of a key gene in ABA biosynthesis, *NCED3* (Alcázar et al. 2006, 2010b). The catabolism of PAs was also linked to drought and osmotic stress (Aziz et al. 1998; Toumi et al. 2010). Indeed the two enzymes of the PA catabolic pathway, DAO and PAO, play a role in the ABA signaling pathway in stomata closure (Lie et al. 2000; An et al. 2008). *AtPAO2*, *AtPAO3*, and *AtPAO4* expression in *Arabidopsis* is induced by ABA (Moschou et al. 2008a; Toumi et al. 2010). Activity of CuAO in *Vicia faba* guard cells is an important source for $H_2O_2$ production in ABA-induced stomatal closure (An et al. 2008). Spd could modulate $H_2O_2$ levels in barley seedlings under drought stress, through amine oxidase activity, and in water-stressed cucumber leaves (Kubis 2003, 2008). When drought-tolerant and drought-sensitive lines of grapevine were compared, an increase in PAO activity was observed in the tolerant line (Toumi et al. 2010). Based on their results Toumi et al. (2010) proposed an involvement of $H_2O_2$ production via PAs, DAO, and PAO activity in the ABA pathway.

## 13.4 High-Salinity Stress

Soil salinity is a growing problem around the world, especially in farmlands. Under saline conditions most plants cannot develop well and seed germination is inhibited (Al-Karaki 2001). PAs were identified as important factors in salt tolerance of plants (Krishnamurthy and Bhagwat 1989; Alcázar et al. 2006, 2010b; Groppa and Benavides 2008). In several crop species polyamine levels changed with salinity; in most cases, Put decreased while Spd and/or Spm increased (Zapata et al. 2004).

The results showed that accumulation of PAs under salt stress is a common phenomenon in plants, and in most cases enhanced activity of *ADC2* and also amine oxidase seems to be important. In *Arabidopsis*, the expression of *ADC2* and *SPMS* was increased under high salinity (Soyka and Heyer 1999), and *ADC2* is required for Put accumulation in salt tolerance (Urano et al. 2004). Treatment with Put ameliorated the salinity effect in salt-tolerant rice (Krishnamurthy 1991). For the same species a protective role could also be demonstrated for Spd by exogenous application (Roy et al. 2005). Mutant plants unable to synthesize Spm were hypersensitive to NaCl and the effect could be cured by application of Spm (Yamaguchi et al. 2006). Transgenic approaches that increased endogenous PA levels in rice enhanced salt-stress tolerance (Roy and Wu 2001, 2002). Not only altered biosynthesis of PAs but also changes in catabolism could be demonstrated in response to salt stress (Aziz et al. 1998; Cona et al. 2006). Catabolism of PAs through the activity of DAO/CuAO and PAO produces $H_2O_2$, which increases the expression of genes involved in salt tolerance, as was shown for tobacco plants that overexpressed maize *ZmPAO* where higher amounts of $H_2O_2$ were produced with increased Spd levels (Moschou et al. 2008b). A positive correlation of PAO activity to salt tolerance was also shown in maize. Under high salinity, reactive oxygen species (ROS) produced by PAO activity in the apoplast sustain maize leaf growth (Rodríguez et al. 2009). CuAO-mediated PA degradation produces γ-aminobutyric acid (GABA), which plays a critical role in salinity stress (Xing et al. 2007). Besides GABA, nitric oxide (NO), another signaling molecule in plants (Palavan-Unsal and Arisan 2009), was shown to be linked to PA metabolism in *Arabidopsis* in that PAs induced the production of NO (Tun et al. 2006). Involvement of *AtCuAO1* in ABA-induced NO production suggests CuAO is an intermediate signal component of ABA-mediated environmental stress responses (Wimalasekara et al. 2011). Gupta et al. (2013) recently combined the data available for PAs, DAO, PAO, and NO in salt stress, and speculated that PA-induced NO generation, possibly through DAO and PAO activity, might be an intermediate candidate involved in salt-stress tolerance. In plants, the salt overly sensitive (SOS) signaling pathway that comprises *SOS3*, *SOS2*, and *SOS1* has been proposed to mediate cellular signaling under salt stress (Mahajan et al. 2008; Ji et al. 2013). Alet et al. (2012) investigated the long-term acclimation of salt-hypersensitive SOS mutants of *Arabidopsis* and PA biosynthesis mutants to salt stress. They found that the accumulation of free Spd and Spm was independent of the SOS signaling pathway and thus might be a different response to high-salinity stress.

## 13.5 Biotic Stress

Plants are permanently threatened by potential pathogens in the air and in the soil. To survive, plants must sense the presence of the invaders by a stimulus to initiate an effective defense against them (Chisholm et al. 2006; Jones and Dangl 2006; Nishimura and Dangl 2010). Involvement of PAs and their metabolism in the defense responses against diverse pathogens has been demonstrated. PA accumulation and further oxidation by DAOs and PAOs were found to play a key role in plant defense

against viruses, bacterial, fungal, and oomycete pathogens (Greenland and Lewis 1984; Walters et al. 1985; Torrigiani et al. 1997; Walters 2003a, b; Asthir et al. 2004; Marina et al. 2008; Mitsuya et al. 2009; Moschou et al. 2009; Sagor et al. 2009; Gonzalez et al. 2011). A role especially of Spm in pathogen defense has been depicted. Spm treatment could activate a subset of hypersensitive response (HR)-specific genes as a consequence of a mitochondrial dysfunction induced via a signaling pathway that stimulates two important mitogen-activated protein kinases (MAPK) that are involved in plant defense, wound-induced protein kinase (WIPK) and salicylic acid (SA)-induced protein kinase (SIPK) (Takahashi et al. 2003, 2004). Spm also induces the expression of a number of *Arabidopsis* genes in cucumber mosaic virus (CMV) infection and was proposed to play a role in signaling defense responses of *Arabidopsis* against this pathogen (Mitsuya et al. 2009; Sagor et al. 2009). Earlier it was found that Spm induced acidic pathogenesis-related protein expression in tobacco mosaic virus (TMV)-infected tobacco plants (Yamakawa et al. 1998). Manipulation of Spm levels through modifications of *SPMS* gene expression can modulate resistance of *Arabidopsis* to the bacterial pathogen *Pseudomonas viridiflava* (Gonzalez et al. 2011). The foregoing results mean that Spm has a signaling function to induce a defense reaction against viral pathogens, and therefore a specific Spm-signaling pathway in the pathogenic stress response of plants was proposed (Mitsuya et al. 2007, 2009; Uehara et al. 2005). However, the mechanism of Spm as an elicitor of plant defense response requires further research.

As for abiotic stresses, a pivotal role of PA catabolism with participation of PAOs and their reaction products, $H_2O_2$ or the aldehyde, in plant–pathogen interaction is evident (Moschou et al. 2009). PAO-derived $H_2O_2$ triggers HR in tobacco plants upon TMV infection and treatment with cryptogein, a proteinous elicitor secreted by the oomycete *Phytophthora cryptogea*, respectively (Yoda et al. 2003, 2006, 2009). Tobacco plants infected by either the nectrotrophic fungus *Sclerotinia sclerotiorum* or by the biotrophic bacterium *Pseudomonas viridiflava* enhance PA levels, leading to increased necrosis through PA oxidation, which plays a detrimental role for the nectrophic fungus although it is beneficial for the biotrophic bacterium (Marina et al. 2008). When T-Spm was exogenously applied to *Arabidopsis* or the T-Spm synthase gene, *ACL5* was ectopically expressed in plants that were more resistant to biotrophic bacteria. The phenomenon was blocked by application of a PAO inhibitor, suggesting the participation of T-Spm oxidation (Marina et al. 2013). Exogenously applied T-Spm restricts CMV multiplication via induced expression of a subset of pathogen-responsive genes in *Arabidopsis* (Sagor et al. 2012b). Recently, these authors showed that longer uncommon polyamines (LUPAs) such as caldopentamine, caldohexamine, homocaldopentamine, and homocaldohexamine have similar activity in the control of the viral pathogen (Sagor et al. 2013).

A pivotal role in the PA-related pathogen defense reaction was also assigned to arginine decarboxylase (ADC) as the key enzyme of PA biosynthesis. The expression of both *ADC1* and *ADC2* increased during the hypersensitive response triggered by avirulent CMV infection on *A. thaliana* (Mitsuya et al. 2009). Also, infection of *A. thaliana* roots by the cist nematode *Heterodera schachtii* increased the expression of both ADC genes, along with other genes involved in polyamine biosynthesis (Hewezi et al. 2010). A predominant role of *ADC* has also been found

in the interaction of maize and the biotrophic pathogenic fungus *Ustilago maydis*, leading to the conclusion that PAs, mainly Put, might play an active role in this pathosystem (Rodríguez-Kessler et al. 2008). The role of Put was investigated in the response of *A. thaliana* to *Pseudomonas syringae* pv. tomato DC3000 infection in connection with MAPK cascades (Kim et al. 2013b). In this system the expression of *ADC2* was highly induced whereas the transcript levels of *ADC1* were slightly upregulated. Recently, another group (Kim et al. 2013a) used the pepper *Xanthomonas campestris* pv. vesicatoria (Xcv) pathosystem to identify pepper ADC1 (CaADC1) as protein that interacts with the effector protein AvrBsT. Transient coexpression of CaADC1 with avrBsT in *Nicotiana benthamiana* leaves specifically enhanced AvrBsT-triggered cell death, accompanied by an accumulation of PAs, NO, and $H_2O_2$ bursts. Among the PAs, Spm application strongly induced NO and $H_2O_2$ bursts, ultimately leading to cell death. The authors suggest that CaADC1 may act as a key defense and cell death regulator via mediation of PA and GABA metabolism (Kim et al. 2013a). This was the first observation that ADC directly interacts with a component of the plant pathogen defense system and emphasizes the key role of ADC in the plant stress response.

## 13.6 Conclusions

As summarized in this chapter, an enhanced stress tolerance of plants can be connected with a protective effect of polyamines using exogenous application or by manipulation of polyamine biosynthetic and metabolic pathways using genetic engineering. Arginine decarboxylase (ADC) as the key enzyme of PA biosynthesis plays a pivotal role in these PA-involved stress responses. Taking all the current knowledge and future perspectives into account, polyamines could function as ideal targets for future crop improvement in changing environments. However, the underlying mechanism of PA-mediated plant stress responses needs to be further dissected.

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# Part VI
# Polyamine Transporters

# Chapter 14
# Recent Advances in Bacterial Polyamine Transport Systems

**Shin Kurihara and Hideyuki Suzuki**

**Abstract** Many bacteria take up external polyamines to optimize their growth and adaptation to the environment. Furthermore, cell-to-cell communication using polyamines recently reported requires export of polyamines and uptake or recognition of polyamine outside the cell. At physiological pH, polyamines are positively charged and hydrophilic and therefore cannot pass through hydrophobic cytoplasmic membranes. Consequently, a polyamine transporter is required for their uptake and export in bacteria. Seven polyamine transporters, that is, PotABCD, PotE, PotFGHI, PuuP, PlaP, CadB and MdtJI, have been reported in *Escherichia coli*, in which polyamine transporters have been well studied since the 1990s. Recently, a growing body of research on polyamine transporters of other bacteria, especially pathogenic bacteria such as *Proteus mirabilis*, *Vibrio cholerae*, *Aggregatibacter actinomycetemcomitans*, and *Streptococcus pneumoniae,* is underway guided by current understanding about polyamine transporters of *E. coli*.

In this chapter, recent understanding in bacterial polyamine transport is outlined in addition to the overview of polyamine transporters in *E. coli*.

**Keywords** CadB • MdtJI • Pathogenic bacteria • PlaP • Polyamine • PotABCD • PotE • PotFGHI • PuuP • Transporter

## 14.1 Introduction

Polyamines are biogenic compounds required for the maintenance of life and environmental adaptation. However homology searches based on previously identified polyamine biosynthetic proteins indicate that some bacteria do not have a

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biosynthetic pathway for polyamines. These bacteria must import polyamines from outside to optimize their growth and adaptation to the environment. Even for bacteria possessing a polyamine biosynthetic pathway, uptake of polyamines is economical because *S*-adenosylmethionine (AdoMet), whose biosynthesis requires ATP, is consumed in the AdoMet decarboxylase (SpeD)-spermidine synthetase (SpeE) pathway.

At physiological pH polyamines are positively charged and hydrophilic and therefore cannot pass through hydrophobic cytoplasmic membranes. Consequently, a polyamine transporter is required for their uptake in bacteria. Bacterial polyamine transporters have been under investigation for many years using *Escherichia coli*. Recently, as cell-to-cell communication using polyamines attracts more attention, a growing body of research on polyamine transporters of other bacteria, especially pathogenic bacteria, is underway guided by current understanding about polyamine transporters of *E. coli*.

In this chapter, recent understanding in bacterial polyamine transport systems is outlined in addition to an overview of polyamine transporters in *E. coli*.

## 14.2 Polyamine Transporters in *E. coli*

Seven polyamine transporters have been reported in *E. coli* that can be classified into four groups.

1. ABC (ATP binding cassette) type polyamine importers: PotFGHI for putrescine and PotABCD for spermidine.
2. Putrescine importers driven by proton motive force: PuuP and PlaP.
3. Polyamine-amino acid antiporters: PotE, putrescine-ornithine antiporter; CadB, cadaverine-lysine antiporter.
4. Spermidine exporter: MdtJI.

It is thought that *E. coli* uses these transporters according to environmental conditions and growth phase.

### *14.2.1 PotFGHI*

PotFGHI is a putrescine importer discovered by Pistocchi and Igarashi et al. in 1993 (Pistocchi et al. 1993). It is an ABC transporter consisting of a periplasmic substrate-binding protein for putrescine (PotF), membrane-associated ATPase (PotG), and two transmembrane channel-forming proteins (PotH and PotI). There is feedback inhibition by intracellular spermidine and putrescine in the PotFGHI uptake system (Terui et al. 2014). Additionally, *potFGHI* is not induced by putrescine and is not repressed by glucose, suggesting that PotFGHI functions for rapid growth with glucose (Terui et al. 2014).

### 14.2.2 PotABCD

PotABCD is a spermidine importer discovered by Furuchi and Igarashi et al. in 1991 (Furuchi et al. 1991) and is a member of the ABC superfamily of transporters. It consists of a membrane-associated ATPase (PotA), two transmembrane channel-forming proteins (PotB and PotC), and a periplasmic substrate-binding protein for spermidine (PotD). In *E. coli*, uptake of spermidine by PotABCD is important for the induction of surface motility (Kurihara et al. 2009a). PotD has similarity to PotF, the putrescine binding protein of the PotFGHI system, and in vitro assays showed that PotABCD took up putrescine in addition to spermidine (Furuchi et al. 1991). $Glu^{171}$, $Trp^{255}$, and $Asp^{257}$ are important for binding activity of PotD to spermidine, and mutations of these amino acid residues resulted in severely reduced spermidine transport activity of PotABCD (Igarashi and Kashiwagi 1999).

### 14.2.3 PuuP

PuuP, a proton-dependent putrescine importer discovered by Kurihara and Suzuki et al. in 2005 (Kurihara et al. 2005), is located in a catabolic gene cluster encoding enzymes for metabolism of putrescine as a nitrogen or carbon source. When *E. coli* grows on putrescine as a sole carbon or nitrogen source, PuuP is the most important importer of the putrescine transporters (Kurihara et al. 2009b). There are differences between the two putrescine transporters, PuuP and PotFGHI (Terui et al. 2014):

1. PotFGHI is feedback inhibited by intracellular polyamine but PuuP is not.
2. PotFGHI is not induced by putrescine supplemented into the growth medium whereas PuuP is induced by putrescine.
3. PotFGHI is not repressed by glucose supplementation but PuuP is repressed. PuuR (Nemoto et al. 2012), whose gene is encoded in the cluster where *puuP* is located, controls this repression and induction (Terui et al. 2014).

### 14.2.4 PlaP

PlaP is a proton-dependent putrescine importer discovered by Kurihara et al. in 2011 (Kurihara et al. 2011) that is important when *E. coli* exhibits surface motility induced on LBGS plates [LB (BD) containing 0.5 % glucose and 0.6 % Eiken agar]. In this culture condition, PlaP is the main transporter for putrescine because other putrescine transporters are repressed (Kurihara et al. 2011). PlaP is a homologue (63 % identity) of PuuP but the $K_m$ value of PlaP for putrescine is 50 times higher than that of PuuP (Kurihara et al. 2011). It was previously reported that the amount of putrescine in culture exported by *E. coli* increased roughly in proportion to the degree of growth (Schiller et al. 2000). The extremely low affinity of PlaP for

putrescine may allow *E. coli* to sense cell density depending on the concentration of extracellular putrescine, because PlaP cannot decrease extracellular putrescine effectively when the concentration of putrescine is below the $K_m$ value.

### *14.2.5 PotE*

PotE is a putrescine transporter discovered by Kashiwagi and Igarashi et al. in 1992 (Kashiwagi et al. 1992). In neutral pH PotE is a proton-dependent putrescine importer, but in acidic pH PotE is a putrescine-ornithine antiporter. Located in the same operon as *potE* is *speF,* an acid-inducible ornithine decarboxylase. SpeF converts ornithine to putrescine with consumption of a proton, and PotE exports putrescine with the uptake of ornithine. Through this process *E. coli* adapts to the acidic environment (Igarashi and Kashiwagi 2010). Also, in neutral pH, *E. coli* excretes putrescine into environment independently of PotE (Schiller et al. 2000), suggesting that there is another unidentified putrescine exporter of *E. coli*.

### *14.2.6 CadB*

CadB is a cadaverine transporter identified by Soksawatmaekhin and Igarashi et al. in 2004 (Soksawatmaekhin et al. 2004). In neutral conditions, CadB is a proton-dependent cadaverine importer but at acidic pH CadB is a cadaverine-lysine antiporter. As in PotE and SpeF, *cadB* is located in the same operon as *cadA* encoding lysine decarboxylase. When *E. coli* grows in an acidic environment, CadA converts lysine to cadaverine with consumption of a proton and CadB exports putrescine with uptake of lysine. This process also allows *E. coli* to adapt to the acidic environment. The two systems for adaptation to acidic environments, the CadB-cadaverine system and the PotE-putrescine system, are very similar, and when the CadB-cadaverine system is disrupted the PotE-putrescine system is induced (Soksawatmaekhin et al. 2004), suggesting that these two systems are complementary in the adaptation to acidic stress.

### *14.2.7 MdtJI*

Higashi and Igarashi et al. reported in 2008 that MdtJI, which had been annotated as a multidrug exporter, is important for cell viability of an *E. coli* Δ*speG* strain lacking spermidine acetyltransferase, which detoxifies spermidine overaccumulated in the cell, when the Δ*speG* strain was grown in media containing high concentrations (2 or 12 mM) spermidine (Higashi et al. 2008). In this study MdtJI excreted spermidine and the expression of MdtJI was induced by spermidine supplemented into the media.

## 14.3 Distribution of Polyamine Transporters Found in *E. coli*

PotD, a spermidine-binding protein in the PotABCD system, shares 34 % amino zacid sequence identity with PotF, a putrescine-binding protein in the PotFGHI system. PotE shares 28 % amino acid sequence identity with CadB and PuuP shares 62 % amino acid identity with PlaP. Figure 14.1 shows the results of the BlastP (NCBI) analysis of transporter proteins against some major phyla. Proteins with high similarity to PotD and/or PotF are found in many phyla including Proteobacteria, Fusobacteria, Deinococci, Cyanobacteria, Spirochaetes, Planctomycetes, Acidobacteria, Thermotogae, Firmicutes, Tenericutes, and Actinobacteria. In comparison, the distribution of proteins with high similarity to

| Phylum | Proteobacteria | | | | Fusobacteria | Deinococci | Cyanobacteria | Spirochaetes | Fibrobacteres |
|---|---|---|---|---|---|---|---|---|---|
| Class | α–proteobacteria | β–proteobacteria | δ–proteobacteria | ε–proteobacteria | | | | | |
| Order | | | | | | | | | |
| PotF | WP_019646659.1 | WP_010095551.1 | YP_005169080.1* | YP_005554394.1* | WP_005884698.1** | YP_004057618.1 | YP_007117535.1 | WP_002770505.1 | YP_003250396.1 |
| | 441 bits(1135) | 388 bits(997) | 193 bits(491) | 197 bits(502) | 166 bits(420) | 199 bits(505) | 218 bits(554) | 157 bits(398) | 132 bits(332) |
| PotD | WP_021999563.1 | WP_006411182.1** | YP_002436368.1** | YP_005554394.1* | WP_005884698.1** | WP_003045914.1* | YP_008711083.1* | YP_004698774.1** | YP_003250396.1 |
| | 227 bits(708) | 207 bits(528) | 353 bits(907) | 365 bits(936) | 207 bits(528) | 215 bits(547) | 246 bits(628) | 218 bits(556) | 191 bits(484) |
| PuuP | WP_008854235.1 | YP_003278314.1 | WP_005030204.1 | YP_007527117.1 | WP_022486558.1 | WP_019009099.1 | YP_007093905.1 | WP_021917866.1 | YP_003249870.1 |
| | 370 bits (951) | 435 bits (1119) | 244 bits (624) | 97.4 bits (241) | 112 bits(281) | 84.7 bits(208) | 106 bits(265) | 101 bits(251) | 31.6 bits(70) |
| PlaP | WP_008854235.1 | WP_003079205.1 | WP_005030204.1 | YP_007527117.1 | WP_022486558.1 | WP_019009099.1 | YP_007093905.1 | WP_021917866.1 | YP_003250890.1 |
| | 327 bits(838) | 479 bits(1233) | 242 bits(618) | 108 bits(269) | 115 bits(288) | 77.0 bits(188) | 120 bits(300) | 101 bits(252) | 26.6 bits(57) |
| PotE | YP_007616303.1 | WP_008814455.1 | WP_022659510.1 | YP_007527117.1 | WP_023051773.1 | YP_004264186.1 | YP_008713851.1 | YP_008435173.1 | YP_003249461.1 |
| | 562 bits(1449) | 610 bits(1573) | 201 bits(512) | 52.8 bits(125) | 91.3 bits(225) | 81.3 bits(199) | 82.0 bits(201) | 127 bits(319) | 28.1 bits(61) |
| CadB | YP_001523898.1 | WP_008540859.1 | WP_022659510.1 | YP_001357207.1 | WP_023049683.1 | YP_004264186.1 | WP_019501884.1 | YP_007234683.1 | YP_003250545.1 |
| | 323 bits(828) | 624 bits(1609) | 433 bits(1113) | 87.4 bits(215) | 110 bits(274) | 77.8 bits(190) | 102 bits(254) | 88.6 bits(218) | 26.6 bits(57) |

| Phylum | Bacteroidetes | | | | Planctomycetes | Chlamydiae | Acidobacteria | Thermodesulfobacteria | Thermotogae |
|---|---|---|---|---|---|---|---|---|---|
| Class | Bacteroidia | Flavobacteriia | Sphingobacteriia | Cytophagia | | | | | |
| Order | | | | | | | | | |
| PotF | WP_022321674.1 | WP_017407209.1 | WP_008245614.1 | YP_004055046.1 | YP_003370470.1 | WP_006342295.1 | WP_020720491.1** | YP_004626973.1 | WP_006489464.1* |
| | 134 bits(338) | 40.8 bits(94) | 32.7 bits(73) | 35.0 bits(79) | 165 bits(417) | 165 bits(417) | 177 bits(449) | 116 bits(291) | 183 bits(464) |
| PotD | WP_022054220.1** | WP_017405960.1 | YP_005008008.1 | WP_009184808.1 | YP_003370470.1 | WP_006342295.1 | YP_008863069.1 | YP_004626973.1 | NP_229176.1 * |
| | 150 bits(378) | 46.2 bits(108) | 32.0 bits(71) | 49.3 bits(116) | 205 bits(522) | 179 bits(455) | 225 bits(574) | 134 bits(336) | 224 bits(571) |
| PuuP | YP_002309295.1 | WP_023578787.1 | WP_008587003.1 | WP_019948034.1 | WP_007220189.1 | YP_004671525.1 | WP_020717260.1 | YP_004628091.1 | YP_002939850.1 |
| | 108 bits(271) | 103 bits(257) | 267 bits(682) | 110 bits(276) | 118 bits(296) | 75.5 bits(184) | 263 bits(671) | 103 bits(258) | 67.4 bits(163) |
| PlaP | WP_022507596.1 | YP_004046406.1 | WP_008587003.1 | WP_019948034.1 | WP_020465840.1 | YP_004671525.1 | WP_020717260.1 | WP_022855826.1 | YP_002939850.1 |
| | 111 bits(277) | 108 bits(269) | 267 bits(682) | 112 bits(279) | 117 bits(294) | 73.2 bits(178) | 255 bits(652) | 93.2 bits(230) | 66.2 bits(160) |
| PotE | WP_019539317.1 | YP_003861715.1 | YP_006255405.1 | WP_009194042.1 | YP_004180681.1 | YP_004671596.1 | BAL54943.1 | WP_022856180.1 | YP_005097361.1 |
| | 96.3 bits(238) | 192 bits(487) | 193 bits(490) | 89.4 bits(220) | 67.4 bits(163) | 211 bits(538) | 88.2 bits(217) | 44.3 bits(103) | 112 bits(280) |
| CadB | WP_019539317.1 | YP_003861715.1 | YP_006255405.1 | YP_006874834.1 | WP_010582070.1 | YP_004671596.1 | YP_827028.1 | YP_004625772.1 | YP_005097361.1 |
| | 103 bits(256) | 189 bits(479) | 216 bits(551) | 85.9 bits(211) | 57.0 bits(136) | 224 bits(570) | 104 bits(259) | 42.0 bits(97) | 130 bits(328) |

| Phylum | Firmicutes | | | | Tenericutes | Actinobacteria | | Dictyoglomi |
|---|---|---|---|---|---|---|---|---|
| Class | Bacilli | | Clostridia | Erysipelotrichi | | Actinobacteria | | |
| Order | Lactobacillales | Bacillales | | | | Actinomycetales | Bifidobacteriales | |
| PotF | ERE64013.1 | WP_008174091.1* | YP_003936770.1** | WP_006783176.1** | WP_021962053.1 | YP_007041178.1** | WP_007593886.1 | YP_002352821.1 |
| | 702 bits(1811) | 196 bits(497) | 191 bits(485) | 164 bits(416) | 150 bits(379) | 205 bits(521) | 140 bits(352) | 27.3 bits(59) |
| PotD | ERE64783.1* | YP_004567724.1* | WP_021803570.1 | WP_009607738.1** | WP_021962053.1 | WP_016697272.1** | WP_007593886.1** | YP_002251767.1 |
| | 669 bits (1725) | 269 bits(687) | 271 bits(692) | 199 bits(505) | 228 bits (581) | 193 bits(490) | 174 bits(440) | 30 bits (66) |
| PuuP | ERE47320.1 | WP_000521245.1 | YP_004497140.1 | WP_006525734.1 | WP_010320904.1 | WP_022908428.1 | WP_022860957.1 | YP_002250776.1 |
| | 828 bits(2138) | 562 bits(1448) | 274 bits(700) | 68.9 bits(167) | 40.8 bits(94) | 439 bits(1128) | 339 bits(869) | 27.7 bits(60) |
| PlaP | ERE52060.1 | WP_002064395.1 | WP_018086197.1 | WP_006525734.1 | YP_003559846.1 | WP_020501606.1 | WP_022860957.1 | YP_002250227.1 |
| | 833 bits(2152) | 504 bits(1299) | 261 bits(667) | 88.2 bits(217) | 47.0 bits(110) | 419 bits(1077) | 304 bits(778) | 26.6 bits(57) |
| PotE | CAM07323.1 | WP_002494724.1 | WP_021655280.1 | WP_018580315.1 | WP_021962055.1 | WP_016663911.1 | WP_022859529.1 | YP_002352430.1 |
| | 565 bits(1456) | 532 bits(1371) | 624 bits(1610) | 96.3 bits(238) | 82.0 bits(201) | 546 bits(1406) | 76.3 bits(186) | 29.3 bits(64) |
| CadB | ERE45997.1 | WP_002498214.1 | WP_021655280.1 | WP_003774312.1 | YP_005907540.1 | WP_018534269.1 | WP_022859528.1 | YP_002352780.1 |
| | 843 bits(2179) | 202 bits(515) | 192 bits(487) | 89.4 bits(220) | 70.5 bits(171) | 177 bits(448) | 103 bits(258) | 28.1 bits(61) |

**Fig. 14.1** Distribution of homologues of polyamine transporters identified in *Escherichia coli*. Amino acid sequences of transporters previously identified in *E. coli* were analyzed using NCBI BLAST. The algorithm was blastp. The database used was non-redundant protein sequence (nr). Taxonomic filters: phylum, class, or order, were used depending on the frequency of protein possessing high similarity to the protein in *E. coli*, in the taxon. Proteins having the highest similarity in the BLAST analyses in the taxonomic filters were listed. In the respective matrixes *above* is sequence IDs and *below* is bit values showing the similarity. The matrixes for proteins whose bit values were from 150 to 200, from 200 to 300, or over 300 were filled with *light grey*, *dark grey*, or *black*, respectively. If all three important residues,: $Glu^{171}$, $Trp^{255}$, and $Asp^{257}$, for recognition of spermidine by PotD, are conserved, the proteins are shown with an *asterisk*. If one or two of these important amino acid residues for the recognition of spermidine by PotD is/are similar residue(s), the proteins are shown with *double asterisk*

PotE and/or CadB is limited to phyla Proteobacteria, a part of Bacteroidetes, Chlamydiae, Firmicutes, and Actinobacteria. Similarly, compared to this, the distribution of proteins with high similarity to PuuP and/or PlaP is limited to phyla Proteobacteria, Sphingobacteria, Acidobacteria, Firmicutes, and Actinobacteria. It is interesting to note that six polyamine transporters possessed by *E. coli* are also frequently found in phylum Firmicutes, especially class *Bacilli*, which is phylogenetically distant from *E. coli*. Although there are many reports studying polyamine transporter homologues of *E. coli* in other bacteria, only one completely novel polyamine transporter, Blt of *Bacillus subtilis*, a spermidine exporter identified by Woolridge and Neyfakh (Woolridge et al. 1997), has been discovered outside *E. coli*. For a better understanding of bacterial polyamine transport, it is important that novel polyamine transporters in bacteria other than *E. coli* are identified.

## 14.4 Studies on Pathogenic Bacteria and Polyamine Transporters

In 2004 Sturgill and Rather reported that putrescine biosynthesis and putrescine as a cell-to-cell signaling molecule are important for differentiation of swarmer cells, which are required for swarming motility in uropathogenic *Proteus mirabilis* (Sturgill and Rather 2004). Because several virulence factors produced by *P. mirabilis* are coordinately regulated with swarmer cell differentiation, a relationship between polyamine and the expression of pathogenicity was suggested. Consequently, much research was undertaken to examine the relationship between pathogens and polyamines. Although most of this research was conducted on polyamine biosynthesis (Patel et al. 2006; Lee et al. 2009; Nasrallah et al. 2011), some research was focused on polyamine transporters.

The causative organism of the disease cholera, *Vibrio cholerae*, possesses three PotD homologues: NspS, PotD1, and PotD2. Karatan et al. reported that *V. cholerae* biofilm formation was regulated positively by extracellular norspermidine sensed by NspS (Karatan et al. 2005). On the other hand, McGinnis et al. reported that *V. cholerae* biofilm formation was regulated negatively by extracellular spermidine imported by a transport system including PotD1 (McGinnis et al. 2009). Additionally, proteomics analysis revealed that the secretion of PotD into environment is correlated with the formation of biofilms in *Aggregatibacter actinomycetemcomitans*, causing aggressive periodontitis (Zijnge et al. 2012). Furthermore, Shah and Swiatlo et al. reported that in *Streptococcus pneumoniae*, which causes pneumonia, uptake of spermidine by PotABCD is important for pathogenicity in mice, and an anti-PotD antibody was used in a study of infection control of *S. pneumoniae* (Shah and Swiatlo 2006). In uropathogenic *Proteus mirabilis* PlaP is important for putrescine uptake, swarming motility, and urothelial cell invasion. Furthermore, the putrescine transport inhibitor triamide-44 inhibits these processes (Kurihara et al. 2013).

Bacteria and animals use ornithine decarboxylase in polyamine biosynthesis. However, the transport system for polyamines in bacteria through transporters is different from that of animals through endocytosis (Uemura et al. 2010). Some bacteria and most animals use ornithine decarboxylase for polyamine biosynthesis. However, the polyamine transport systems in bacteria are different from those of animals, which employ endocytosis (Uemura et al. 2010). Therefore, in view of the potential adverse effects that could result from the inhibition of polyamine biosynthesis in human, using an inhibitor of polyamine transport as a drug for prevention and control of pathogenic bacteria is a more attractive chemotherapeutic target than polyamine biosynthesis.

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# Chapter 15
# Polyamine Transport Systems in Plants

**Miki Fujita and Kazuo Shinozaki**

**Abstract** Polyamine (PA) transport has been analyzed at the cell and organ levels in several plant species, but currently the molecular mechanisms of PA transport are not completely understood. Several papers recently identified plant PA transporters. A study of *Arabidopsis* oxidative stress responses to the herbicide paraquat (PQ) identified a LAT (L-type amino acid transporter) family protein as a transporter of both PQ and PA. Other studies based on yeast complementation analyses revealed that LAT family genes in rice and *Arabidopsis* function as PA transporters. *Arabidopsis* LAT proteins exhibited different subcellular localizations, suggesting that these transporters could be involved in both intracellular PA trafficking and PA uptake across the plasma membrane. These results provide novel insights into plant PA transport and explain the mechanism for PA-mediated protection against PQ toxicity. In this review, we focus on recent advances in our understanding of plant PA transport systems and discuss the roles of the recently identified plant PA transporters.

**Keywords** Arabidopsis • Paraquat • Plant • Polyamine • Rice • Transporter

## 15.1 Introduction

Polyamines (PAs) are essential for plant growth and development. PAs have multiple functions in numerous biological processes, and the possibility that PAs act as phytohormones or second messengers has been discussed. An important criterion for phytohormones is translocation from the site of synthesis to other sites where their function is exerted. Therefore, PA transport systems have been a subject of interest. Although the major source of PAs is thought to be via de novo biosynthesis, PA uptake and transport are also considered to have significant roles in PA homeostasis (Abdulhussein and Wallace 2014; Igarashi and Kashiwagi 2010). In plant cells, PAs are localized in cell walls, vacuoles, nuclei, mitochondria, and chloroplasts (Slocum and Flores 1991). This biosynthetic compartmentalization

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suggests that intracellular transport is important for the physiological role of PAs. In plant studies, PA transport was reported for the first time in apples. Exogenously applied putrescine (Put) was absorbed by apple leaves and flowers, and subsequently affected fruit set, fruit growth, and yield (Costa and Bagni 1983; Bagni et al. 1984). Subsequent studies in diverse types of plants also reported that exogenously applied PAs, including spermine (Spm), spermidine (Spd), and Put, elicit a wide range of biological responses such as development, fertilization, senescence, and stress responses (Busch et al. 1996). These results support the existence of PA transport systems in plants.

Multiple PA transporters have been identified in bacteria and yeast (see Chap. 14 in this volume for details) (Igarashi and Kashiwagi 2010; Kashiwagi and Igarashi 2011). Recent reports provide molecular details of PA transport in eukaryotic cells (Abdulhussein and Wallace 2014; Poulin et al. 2012). Mammalian SLC3A2, which encodes the heavy subunit of heteromeric amino acid transporter (HAT), has been identified as a diamine exporter that has the ability to export Put (Pottosin et al. 2012). Two members of the SLC22A family proteins, OCT1-3 (SLC22A1-3) and OCT6 (SLC22A16), were previously identified as a passive diffusion organic cation carrier and a carnitine/cation transporter, respectively. Recent work shows that OCT1-3 and OCT6 are also involved in PA transport (Abdulhussein and Wallace 2014; Busch et al. 1996; Grundemann et al. 2003; Winter et al. 2011). These plasma membrane transporters are suggested to mediate PA transport followed by vesicular sequestration. Additionally, two types of endocytosis have been implicated in PA transport (Poulin et al. 2012; Soulet et al. 2004). In recent years, plant transporters involved in PA transport have been identified. In this chapter, we discuss recent progress and the current understanding of PA transport in plants.

## 15.2 Subcellular Localization and Transport of Polyamines in Plants

In plant cells, PAs are localized primarily in the cell wall and vacuoles (Edreva 1996; Wipf et al. 2002; Goldberg and Perdrizet 1984), and to a lesser extent in mitochondria and chloroplasts (Torrigiani et al. 1986; Pistocchi et al. 1990). The mechanisms and kinetics of PA transport have been studied in protoplasts, vacuoles, and mitochondria using different plant species (reviewed by Kakkar et al. 1998). In carrot, uptake of Put and Spd was very rapid in cultured cells, protoplasts, and vacuoles, and the existence of an antiport mechanism was suggested (Pistocchi et al. 1987). The concentration-dependent uptake of PAs into carrot vacuoles was biphasic, consisting of linear and saturable phases, with $K_m = 61.8$ μM (Pistocchi et al. 1987; Wipf et al. 2002). In mitochondria isolated from Jerusalem artichoke (*Helianthus tuberosus*) tubers, Spm uptake occurred with $K_m = 89$ μM (Pistocchi et al. 1990). In maize roots, Put uptake across the plasma membrane occurred through a saturable, protein-mediated transport system with $K_m = 120$ μM (Ditomaso et al. 1992). Subcellular analysis of maize roots revealed that Put was transported

into vacuoles, and this transport was bidirectional (Ditomaso et al. 1992). These results indicate that PAs are transported into different organelles, suggesting that the subcellular localization of PAs may have a physiological significance.

## 15.3 Identification of RMV1/AtLAT1 as a Paraquat/ Polyamine Transporter

We identified a plant polyamine transporter through an analysis of natural variations in oxidative stress responses (Fujita et al. 2012; Fujita and Shinozaki 2014). We used paraquat (PQ) to induce oxidative stress and isolate genes involved in oxidative stress tolerance. The responses to PQ in wild-type (WT) *Arabidopsis* accessions were compared and analyzed by genome-wide association and F2 mapping. This strategy revealed that the natural variation of PQ tolerance in *Arabidopsis* (WT) accessions was determined by the amino acid polymorphisms of a putative transporter gene. The responsible gene was designated *resistant to methyl viologen 1* (*RMV1*) because methyl viologen is another name for PQ. The *RMV1* gene encodes a putative amino acid permease containing 12 transmembrane domains, which is named AtLAT1 because of sequence similarity with mammalian L-type amino acid transporter (LAT) family proteins. The properties of LAT family proteins in plants have been unclear until recently. The *Arabidopsis rmv1* knockout mutant exhibited greater PQ tolerance than that of the corresponding WT plants, and PQ uptake activity was drastically reduced in *rmv1* mutants. By contrast, PQ uptake was higher in transgenic *Arabidopsis* plants overexpressing *RMV1*. Further analysis of uptake revealed that RMV1-mediated PQ uptake was saturable ($K_m = 24.4$ μM) and dependent on proton cotransport. Polymorphic variations in RMV1 affect PQ uptake activity and confer the natural variations in PQ tolerance observed in *Arabidopsis* accessions. These results indicate that RMV1 is a PQ transporter (Fujita et al. 2012).

Previous studies using various organisms including plants suggested that PQ was transported by carrier systems that regularly mediate PA transport. The competitive interaction between PQ and PA transport was initially demonstrated by studies using rat lung, which aimed to identify compounds that antagonize or inhibit PQ uptake into target tissues and prevent cell damage. These analyses identified a series of diamines and polyamines that were natural substrates for the carrier system that mediated PQ uptake (Smith et al. 1982, 1990). Similar PQ/PA transport competition was observed in *Escherichia coli* (Minton et al. 1990), yeast (Tomitori et al. 1999), and plants (Hart et al. 1992; Kurepa et al. 1998). Therefore, we hypothesized that the plant PQ transporter RMV1 also functioned in PA transport. We constructed transgenic *Arabidopsis* plants overexpressing *RMV1* and tested PA uptake activity. *RMV1*-overexpressing plants were significantly more sensitive to PAs and displayed higher PA uptake activity than that of control lines. The $K_m$ values for uptake of Put, Spd, and Spm were 56.5, 2.2, and 0.6 μM, respectively, and these values were consistent with observations in animal cells showing that the affinity for PA uptake increases in the order Put < Spd < Spm (Seiler and Dezeure 1990).

These results indicate that RMV1 plays a role in both PA and PQ uptake, which is consistent with observations that exogenous application of PAs counteracts PQ toxicity (Fujita et al. 2012; Kurepa et al. 1998).

## 15.4 Identification of Polyamine Transporter Genes Using Yeast Complementation Analysis

Studies using sequence data and yeast mutant complementation analysis identified PA transporters in rice and *Arabidopsis* (Mulangi et al. 2012a, b). Mulangi et al. (2012b) searched for rice and *Arabidopsis* genes that show sequence similarity to PA transporters of *Leishmania major* (*LmPOT1*) (Hasne and Ullman 2005) and *Trypanosoma cruzi* (*TcPAT12*) (Carrillo et al. 2006). Full-length cDNAs of candidate genes were transformed into the *Saccharomyces cerevisiae agp2*Δ mutant, which is deficient in Spd transport (Aouida et al. 2005). The study identified a rice gene designated as *PA uptake transporter 1* (*OsPUT1*), which partially complemented the PA-insensitive *agp2* mutant phenotype. Radiological uptake assays and competitive inhibition analyses in the yeast transformant revealed that OsPUT1 preferentially transported Spd with $K_m = 15.2$ μM (Mulangi et al. 2012b). A similar experimental approach identified five additional PA transporters in rice and *Arabidopsis*, including *OsPUT2*, *OsPUT3*, *AtPUT1*, *AtPUT2*, and *AtPUT3* (Mulangi et al. 2012a). These five PA transporters exhibited affinities to both Put and Spd, with $K_m$ values of 0.94–3.3 μM and 28.7–33.4 μM for Spd and Put uptake, respectively.

*AtPUT3* is identical to the *RMV1/AtLAT1* gene. All *OsPUT* and *AtPUT* genes have been classified as LAT family members. However, a hidden Markov model based on multiple alignments indicated that plant *PUT* genes were not homologous with animal *LAT* genes (Mulangi et al. 2012a), suggesting that they have different functions resulting from low sequence homology. The *AtPUT2/AtLAT4* gene was recently identified as the gene responsible for the *paraquat resistant 1* (*par1*) mutation (Li et al. 2013). The *par1* mutants are resistant to PQ and have lower levels of PQ accumulation in chloroplasts, suggesting that PAR1 is involved in the intracellular transport of PQ into chloroplasts. The same research group also identified the rice gene *OsPAR1* (identical to *OsPUT2*), which regulates rice sensitivity to PQ similarly to that of *PAR1* in *Arabidopsis* (Li et al. 2013). Collectively, *Arabidopsis* and rice *LAT/PUT* genes are involved in PA/PQ transport. *LAT/PUT* family genes have been identified in several plant species, suggesting that PA/PQ transport mediated by LAT/PUT is a common transport pathway in the plant kingdom.

## 15.5 Subcellular Localization of AtLAT/PUT Proteins

RMV1/AtLAT1/AtPUT3 is a plasma membrane-localized protein (Fujita et al. 2012). RMV1-GFP fusion protein localizes to the plasma membrane when transiently expressed in onion epidermal cells or when constitutively expressed in transgenic *Arabidopsis*. By contrast, transient expression analysis using *Arabidopsis*

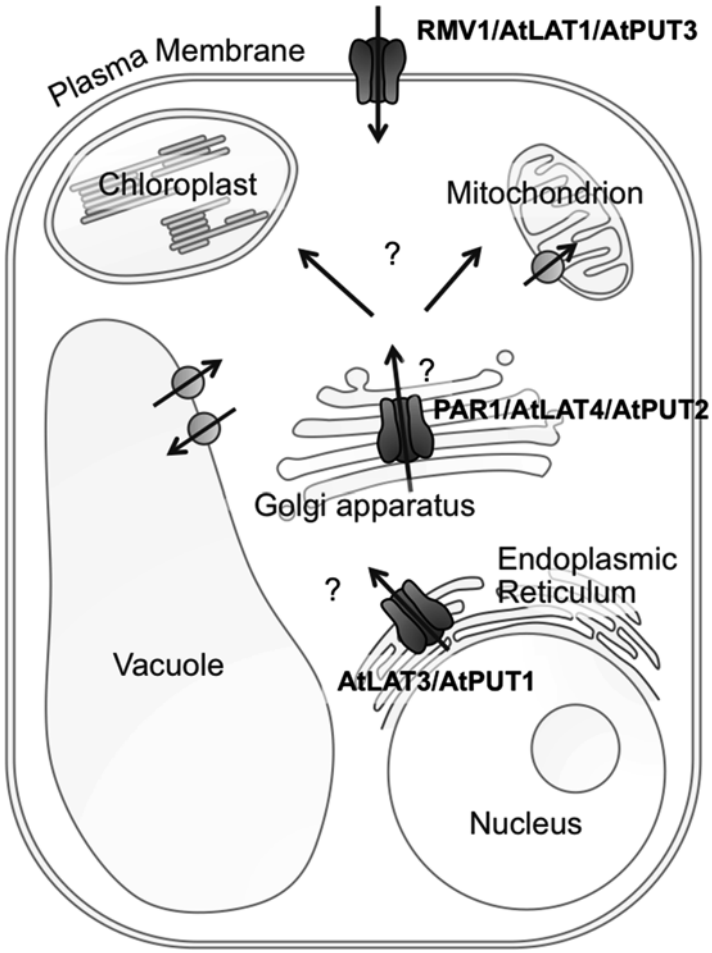


**Fig. 15.1** Schematic illustration of the subcellular localization and substrate preference of known polyamine (PA) transporters in *Arabidopsis*. *Arrows* indicate the transport pathway of PAs. At least three AtLAT/PUT proteins exhibit PA transport activity. The existence of unknown transport systems in vacuoles and mitochondria is suggested

protoplasts indicate that PAR1/AtLAT4/AtPUT2 localizes to the Golgi apparatus, and AtPUT1/AtLAT3 localizes to the endoplasmic reticulum (Li et al. 2013). Rice OsPAR1/OsPUT2 also localizes to the Golgi apparatus (Li et al. 2013). These observations suggest that PUT proteins participate in intracellular PA translocation and PA transport across the plasma membrane (Fig. 15.1).

## 15.6 Perspectives

The recent identification of plant PA transporters is an important step for understanding PA homeostasis in plant cells. Plant LAT/PUT proteins are detected in different subcellular compartments, which may have a role in organellar PA homeostasis. The PA concentrations required to exert biological effects are significantly higher than those of traditionally accepted phytohormones; thus, PAs are not classified as phytohormones. However, PAs play multiple roles in the plant life cycle, although the molecular mechanisms of PA function are still unknown. Future studies using PA transporter knockout mutants and PA biosynthetic mutants will provide valuable information about the biological function of PAs in plants.

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# Part VII
# Polyamine-Derived Secondary Metabolites

# Chapter 16
# Polyamine-Derived Alkaloids in Plants: Molecular Elucidation of Biosynthesis

**Tsubasa Shoji and Takashi Hashimoto**

**Abstract** Alkaloids are nitrogen-containing specialized metabolites generated from primary metabolites, including polyamines, in plants. Alkaloids include many biologically active chemicals and confer adaptive advantages to plants producing them. The tropane alkaloids include cocaine and calystegines, whereas pyrrolizidine alkaloids are a group of defense compounds that contain a necine base moiety derived from homospermidine. Nicotine and tropane alkaloids are commonly derived from the diamine putrescine, which is incorporated into the pyrrolidine ring of nicotine or the tropane ring in tropane alkaloids. Here we review the progress that has been made in understanding the biosynthesis of putrescine-derived alkaloids, which has been intensively studied in tobacco and medicinal plants of the Solanaceae. In solanaceous species, alkaloids, including clinically important hyoscyamine and scopolamine, are synthesized in specific cells in the roots and then translocated to shoots through the xylem. Homospermidine synthase, which catalyzes homospermidine formation from putrescine and spermidine, repeatedly evolved in multiple plant lineages from deoxyhypusine synthase involved in posttranslational activation of eIF5A protein. The evolution of these alkaloid biosynthesis enzymes and pathways is discussed in light of the newly available information in this field.

**Keywords** Nicotine • Putrescine • Pyrrolizidine alkaloids • Solanaceae • Spermidine • Tropane alkaloids

## 16.1 Introduction

In a range of flowering plants, polyamines are metabolized into nitrogen-containing specialized metabolites, including nicotine, tropane, and pyrrolizidine alkaloids. In contrast to primary metabolites, which are essential for growth and exist ubiquitously, these alkaloids are present only in restricted lineages, offering adaptive advantages to the plants that produce them. Biochemical and molecular studies have

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begun to elucidate a number of the enzymes and corresponding structural genes constituting the biosynthetic pathways for polyamine-derived alkaloids (Hashimoto and Yamada 1994; Ober and Kaltenegger 2009; Shoji and Hashimoto 2011a). These pathways begin with the symmetrical diamine putrescine, which also serves the precursor for spermidine and spermine produced by all organisms. Here, we review the current understanding of the biosynthetic pathways for the putrescine-derived alkaloids and their regulation in various biological contexts. The pathways and enzymes covered in this article are shown in Figs. 16.1 and 16.2.

## 16.2 Nicotine

Nicotine is a putrescine-derived alkaloid produced in tobacco (*Nicotiana tabacum*) and related species of *Nicotiana* (Solanaceae). Because of its potent toxicity, the role of nicotine as a defense toxin is well recognized (Steppuhn et al. 2004), and indeed its synthetic analogues, the neonicotinoids, are widely used as insecticides. In *Nicotiana* species, putrescine can be diverted to a pathway specialized for nicotine formation (Fig. 16.1). Putrescine is *N*-methylated by putrescine *N*-methyltransferase (PMT) in the first committed step of alkaloid biosynthesis. PMT shows high sequence similarity to spermidine synthase (SPDS), which is involved in spermidine formation (Hibi et al. 1994; Hashimoto et al. 1998). The similarity of the two proteins implies that PMT evolved from ubiquitous SPDS during the diversification of alkaloid-producing plants. SPDS catalyzes the transfer of the aminopropyl moiety of decarboxylated *S*-adenosylmethionine (dSAM) to putrescine, and PMT transfers the methyl moiety of *S*-adenosylmethionine (SAM) to the same substrate. The switch between the group transferred is based on differential binding of the cosubstrates, dSAM and SAM, and can be achieved with changes of only a few amino acid residues (Junker et al. 2013). *N*-methylputrescine is oxidatively deaminated by *N*-methylputrescine oxidase (MPO) to 4-methylaminobutanal, which cyclizes spontaneously to *N*-methylpyrrolinium cation. MPO protein is a copper- and topaquinone-containing enzyme that belongs to the diamine oxidase (DAO) family (Heim et al. 2007; Katoh et al. 2007). In contrast to typical DAOs that preferentially utilize putrescine, MPO prefers *N*-methylputrescine over non-methylated putrescine, implying that MPOs arose from a DAO by optimizing the substrate specificity, probably via slight changes in amino acid sequence (Naconsie et al. 2014). The *N*-methylpyrrolinium cation, which is common to the nicotine and tropane alkaloid pathways, is a reactive intermediate available for various condensation reactions (Fig. 16.1). The pyrrolinium cation condenses with pyridine ring-containing nicotinic acid or its derivative to produce nicotine, with both pyrrolidine and pyridine rings. It is unknown whether nicotinic acid itself or a derivative is directly incorporated into the pyridine ring of nicotine. Nicotinic acid is a primary metabolite in a pathway supplying nicotinamide adenine dinucleotide (NAD) (Katoh and Hashimoto 2004). Two orphan oxidoreductases of different families, although their exact enzyme activities have yet to be defined, have been suggested as involved in

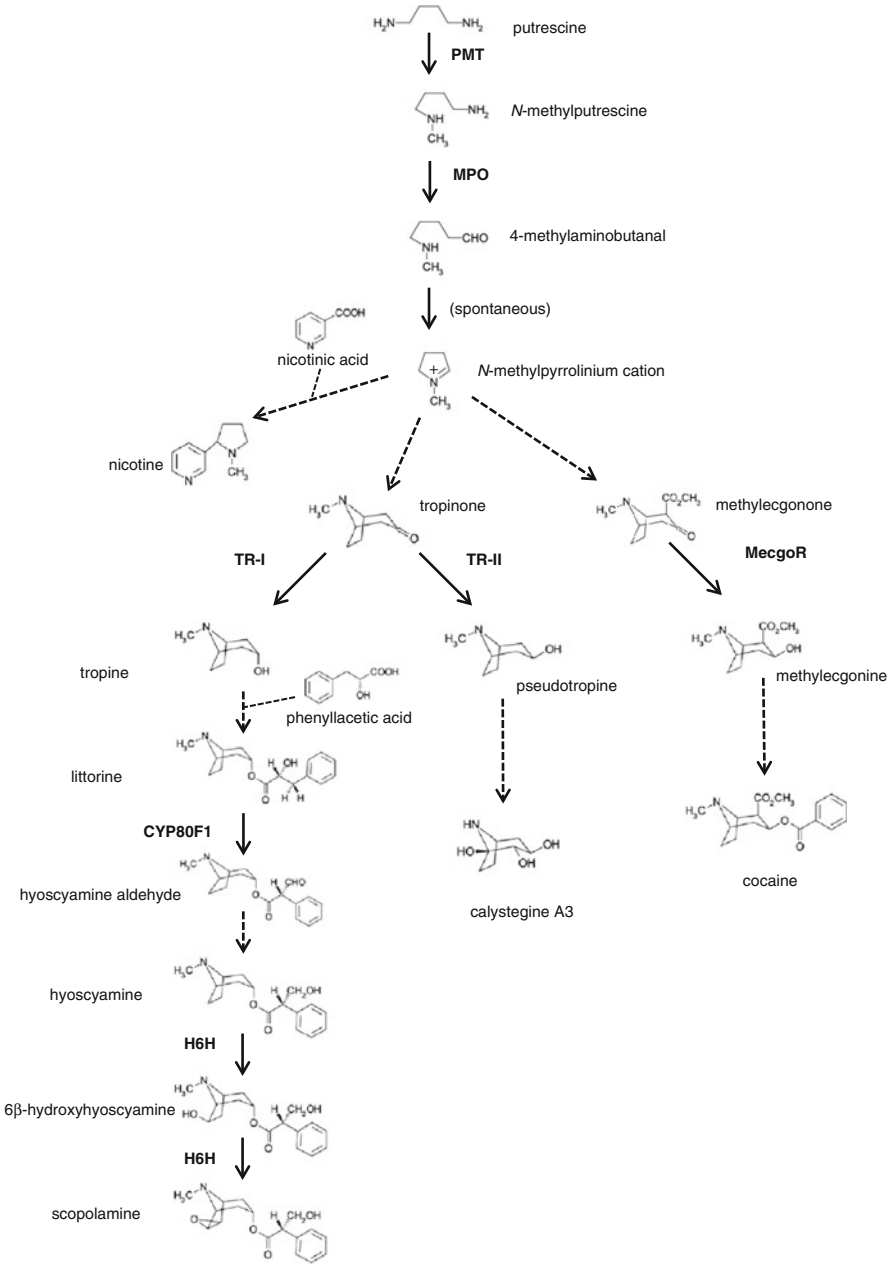


**Fig. 16.1** Biosynthetic pathways for nicotine and tropane alkaloids. Enzymes involved in the pathways are *PMT* putrescine *N*-methyltransferase, *MPO N*-methylputrescine oxidase, *TR-I* tropine-forming tropinone reductase, *TR-II* pseudotropine-forming tropinone reductase, *MecgoR* methylecgonone reductase, *CYP80F1* cytochrome P450 monooxygenase with littorine mutase activity, *H6H* hyoscyamine 6**β**-hydroxylase

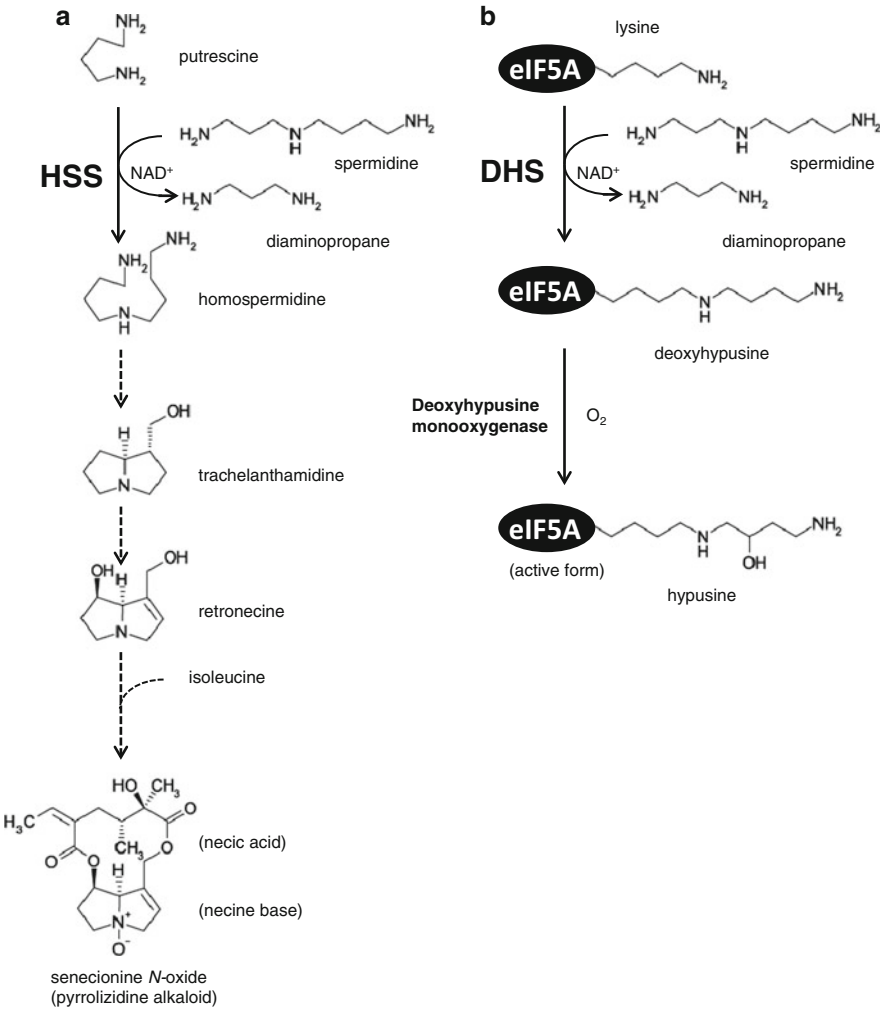


**Fig. 16.2** Pyrrolizidine alkaloid (PA) biosynthesis and posttranslational activation of eIF5A. **a** Homospermidine synthase (HSS) begins PA biosynthesis by forming homospermidine, the first pathway-specific intermediate, which is further metabolized and eventually incorporated into the necine base of PAs. As an example, the generation of senecionine *N*-oxide is shown. **b** In the first of two reactions involved in posttranslational activation of the eIF5A protein, deoxyhupsine synthase (DHS) catalyzes a reaction analogous to that of HSS

the late steps of this pathway, including the ring condensation (Kajikawa et al. 2009, 2011; De Boer et al. 2009).

In tobacco, nicotine is synthesized in the roots and then moves up through the xylem to aerial leaves, where it is stored in central vacuoles. Consistent with root-specific biosynthesis, many biosynthetic genes including *PMT* are expressed in the cortex and endodermis of the roots (Shoji et al. 2000, 2002, 2009; Shoji and

Hashimoto 2011b). In addition, multidrug and toxic compound extrusion (MATE) family transporters localized at the tonoplast are involved in vacuolar sequestration of nicotine (Morita et al. 2009; Shoji et al. 2009). Nicotine production is increased in response to insect attacks, with jasmonate signaling playing a regulatory role in the response. A set of nicotine metabolic and transport genes are directly regulated by ethylene response factor and basic helix-loop-helix transcription factors, which are connected upstream with jasmonate signaling components (Shoji et al. 2010; Shoji and Hashimoto 2011c, 2013).

## 16.3 Tropane Alkaloids

Tropane alkaloids (TAs), a class of specialized metabolites with a bicyclic tropane ring in their structures, include clinically important hyoscyamine and scopolamine, the stimulant and narcotic cocaine, and the nortropane alkaloids calystegines. Plants producing TAs are distributed, sometime sporadically, across separate angiosperm families (e.g., Proteaceae, Convolvulaceae, Brassicaceae, Euphorbiaceae, Rhizophoraceae, Solanaceae, and Erythroxylaceae) (Griffin and Lin 2000). Studies of TA biosynthesis have been performed predominantly in Solanaceae plants, and thus little is known regarding TA pathways in other families.

### *16.3.1 Hyoscyamine and Scopolamine*

The anticholinergic drugs hyoscyamine and scopolamine are produced in certain medicinal plants of the Solanaceae, including species of *Atropa*, *Datura*, *Duboisia*, and *Hyoscyamus*. Atropine is a racemic mixture of naturally occurring (*S*)-hyoscyamine and its *R* isomer, with most of the pharmacological effects attributed to the former. In common with the nicotine biosynthetic pathway, PMT and MPO catalyze early steps of the TA pathway leading to the *N*-methylpyrrolinium cation, which is subsequently incorporated into a tropane ring (Fig. 16.1). *PMT* genes were isolated from TA-producing species based on homology to their tobacco counterparts (Suzuki et al. 1999a), whereas their *MPO* genes, although defined enzymatically (Hashimoto et al. 1990), have yet to be cloned. It is hypothesized that the pyrrolinium cation condenses with acetoacetate and then ring closure occurs after oxidation and another round of aldol condensation (Humphrey and O'Hagen 2001), producing tropinone with a tropane ring. The reduction of tropinone at a keto group is catalyzed by NADPH-dependent tropinone reductases (TRs) of the short-chain dehydrogenase/reductase (SDR) family (Jornvall et al. 1995). Two distinct TRs, TR-I and TR-II, stereospecifically reduce the ketone, generating diastereometric alcohols: tropine with a 3α-configuration and pseudotropine with a 3β-configuration, respectively (Nakajima et al. 1993). The distinct stereospecificities of the reductions are attributed to differences of several amino acid residues between the two TRs,

leading to the opposite orientation of tropinone in substrate-binding cavities (Nakajima et al. 1998, 1999). Tropine is incorporated into hyoscyamine and scopolamine, whereas pseudotropine is converted to calystegines (Fig. 16.1). Tropine is esterified with phenyllacetic acid derived from phenylalanine to generate littorine (Robins et al. 1994). To form hyoscyamine, the phenyllacetate moiety of littorine must be transformed into tropic acid. A cytochrome P450 CYP80F1 with littorine mutase activity is involved in this intramolecular rearrangement, generating hyoscyamine aldehyde from littorine (Li et al. 2006). Subsequently, the aldehyde is converted to hyoscyamine, a reaction that is believed to be catalyzed by an alcohol dehydrogenase. Hyoscyamine then becomes the final product scopolamine via 6β-hydroxyhyoscyamine through hydroxylation and subsequent epoxidation. This two-step reaction is catalyzed by a bifunctional hyoscyamine 6β-hydroxylase (H6H), which belongs to the 2-oxoglutarate-dependent dioxygenase family (Matsuda et al. 1991). Ectopic overexpression of *Hyoscyamus niger H6H* in *Atropa belladonna*, which is normally rich in hyoscyamine because of poor expression of endogenous *H6H*, leads to high accumulation of scopolamine, which is more valuable than hyoscyamine because of its higher pharmacological activity and limited supply (Yun et al. 1992).

TA biosynthesis occurs in roots of plants of the Solanaceae. In *A. belladonna*, both *PMT* and *H6H* genes are specifically expressed in the root pericycle, particularly in cells next to the xylem (Hashimoto et al. 1991; Kanegae et al. 1994; Suzuki et al. 1999a, b). Such proximity of gene expression to the xylem might reflect root-to-shoot translocation of the end product scopolamine through the vasculature tissue connecting the organs. In contrast to PMT and H6H, catalyzing the first and last steps of the pathway, respectively, TR-I protein is localized in the endodermis and cortex, but not in the pericycle, in *H. niger* roots (Nakajima and Hashimoto 1999), suggesting that there might be trafficking of pathway intermediates between tissue layers of the roots to complete TA biosynthesis.

### *16.3.2 Cocaine*

Cocaine is produced in certain species of Erythroxylaceae, such as *Erythroxylum coca*. In *E. coca*, the tropinone reduction step involving conversion of methylecgonone to methylecgonine in the penultimate step of cocaine biosynthesis is catalyzed by NADPH/NADH-dependent methylecgonone reductase (MecgoR), of the aldo-keto reductase (AKR) family (Penning 2004), but not by TR-like SDR proteins (Jirschitzka et al. 2012) (Fig. 16.1). Recombinant MecgoR catalyzes the stereospecific reduction of ketones, namely methylecgonone and tropinone, to the corresponding alcohols with 3β-configuration, methylecgonine and pseudotropine, respectively. In contrast to MecgoR, TR-II of Solanaceae species reduces only tropinone but not methylecgonone (Jirschitzka et al. 2012). In *E. coca*, transcript and protein levels of MecgoR are highest in young leaves, the site of TA production in this species (Docime et al. 2012), but are not detected in the roots, where TRs are expressed in Solanaceae plants. Thus, enzymes of completely different families,

AKD and SDR, have been recruited to carry out similar reduction steps during TA biosynthesis in the Erythroxylaceae and Solanaceae. These enzymes are also differently regulated in the respective species. Together, these findings support the assumption that capacity for TA production has arisen independently in multiple lineages.

### *16.3.3 Calystegines*

Calystegines are a group of polyhydroxylated and nonesterified TAs with a nortropane ring, which is a tropane ring that has been demethylated at a bridge nitrogen (Biastoff and Dräger 2007). The structural similarity of calystegines to monosaccharides allows them to act as selective glycosidase inhibitors with therapeutic potential (Molyneux et al. 1993). Most, but not all, calystegines contain an equatorial hydroxyl group on carbon 3 of the ring, which is a typical feature of pseudotropine. Based on this, calystegines are assumed to be derived from the conventional TA pathway via pseudotropine (Fig. 16.1). This notion is supported by the co-occurrence of calystegines and other TAs in various species (Brock et al. 2005, 2006; Biastoff and Dräger 2007). In potato (*Solanum tuberosum*), calystegines accumulate to a high level in developing sprouts of tubers. In accord with this, *PMT* and pseudotropine-forming *TR-II* are expressed in potato tissues, including the sprouts (Keiner et al. 2002; Stenzel et al. 2006; Kaiser et al. 2006; Richter et al. 2007). Although poorly characterized, pseudotropine is likely subjected to demethylation and hydroxylation to produce calystegines.

## 16.4 Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids (PAs) are constitutively produced in plants as defense compounds against herbivores. Most PAs exhibit hepatotoxicity and carcinogenicity to vertebrates and genotoxicity to insects. In herbivores consuming the plants, PAs are converted by cytochrome P450 enzymes into toxic derivatives that easily react with biological nucleophiles such as proteins and nucleic acids in the cells. Some insects that are specialized to PA-producing plants adapt the alkaloids for their own defense by accumulating them in their bodies (Hartmann and Ober 2008). PAs are distributed in several unrelated families of angiosperms; the vast majority of PAs are found in tribes Senecioneae and Eupatorieae of the Asteraceae, several genera of the Boraginaceae, genus *Crotalaria* of the Fabaceae, and some genera of the Orchidaceae (Hartmann and Witte 1995).

PA consists of a bicyclic necine base moiety that is esterified with necic acid. The symmetrical triamine homospermidine is the first specific intermediate in necine base biosynthesis (Fig. 16.2a). Homospermidine is generated by $NAD^+$-dependent transfer of the aminobutyl group of spermidine to putrescine, catalyzed by homospermidine synthase (HSS). Homospermidine is metabolized through poorly characterized steps and eventually incorporated into the necine base of PAs. HSS is

phylogenetically related to deoxyhypusine synthase (DHS), which is involved in posttranslational activation of eukaryotic initiation factor 5A (eIF5A) (Ober and Hartmann 1999). Utilizing a reaction mechanism analogous to that of HSS but accepting a different substrate, DHS catalyzes the $NAD^+$-dependent transfer of the aminobutyl moiety of spermidine to a side chain of a certain lysine residue in eIF5A, generating the rare amino acid deoxyhypusine (Fig. 16.2b). In the following step, deoxyhypusine is hydroxylated to hypusine by deoxyhypusine monooxygenase. Active eIF5A protein with hypusine is required for cell growth and division (Park et al. 1993), and in plants it functions in developmental processes (Thompson et al. 2004). Of note, DHS protein also accepts putrescine as a substrate to generate homospermidine as a minor side reaction, whereas HSS is unable to bind eIF5A substrate as a substrate and has no DHS activity (Ober et al. 2003). Based on the bifunctional nature of DHS, evolution of HSS from ancestral DHS is assumed to have involved the loss of the DHS protein-binding activity and an increase of the HSS activity that was originally present in DHS at a low level, which might have been favored under selection pressure of herbivory.

To gain insights into HSS evolution in multiple plant lineages, phylogenetic analysis has been performed with HSS and DHS sequences from several PA-producing species. Recruitment of HSS from DHS is assumed to have occurred independently at least five times and repeatedly throughout angiosperm evolution (Reimann et al. 2004; Nurhayati et al. 2009; Kaltenegger et al. 2013). Relaxation of functional constraints during this recruitment is reflected in enhanced substitution rates in HSS sequences and thus longer branches for them in the phylogenetic tree. Expression patterns of HSS have been studied in various PA-producing species. In *Senecio vernalis* (tribe Senecioneae of Asteraceae), *HSS* expression is root specific and restricted to the endodermis and neighboring cortex cells located opposite the phloem (Moll et al. 2002), reflecting phloem-based translocation of PAs in that species. In *Eupatorium cannabinum* (tribe Eupatorieae of the Asteraceae), *HSS* is expressed uniformly in all cells of the cortex but not in the endodermis of the roots (Anke et al. 2004). In *Phalaenopsis* orchids (Orchidaceae), tips of aerial roots and young flower buds are the sites of *HSS* expression (Anke et al. 2008). In three species of Boraginales, each *HSS* is uniquely regulated in the individual species, even though the *HSS* genes share an origin through a single gene duplication event (Niemüller et al. 2012). These studies demonstrate that *HSS* is regulated differently in various lineages, implying that there was divergent evolution of pathway regulation along with that of enzyme structure.

## 16.5 Perspectives

Biochemical and molecular studies have led to the elucidation of a number of key enzymatic steps involved in polyamine-derived alkaloid biosynthesis. Based on their phylogenetic relationships, alkaloid biosynthetic enzymes are presumed to have evolved from structurally related but catalytically distinct proteins, particularly

primary metabolic enzymes, possibly through gene duplications followed by divergence of the new duplicates (Ober 2005). It has been demonstrated for some of the enzymes that the novel enzymatic activities could be generated by a small number of changes in amino acid residues that are critical for the catalytic differences. The expression patterns of some of the biosynthetic enzymes are diverse in various species, implying that the regulation of these enzymes can be flexible. These features—the requirement for a limited number of mutations for enzymatic innovation and the flexibility of regulation—likely underlie the repeated independent evolution of similar alkaloid pathways in multiple plant lineages, as exemplified by the cases of the PA and possibly the TA pathways. Molecular elucidation of the alkaloid pathways in taxonomically distant groups may help to ascertain the polyphyletic origin of these pathways.

A handful of steps remained to be elucidated, such as the ring-coupling steps in nicotine biosynthesis, the steps generating the tropane ring in TA biosynthesis, and the steps following the HSS-catalyzed reaction in PA biosynthesis. Recent development of genomic approaches has advanced our understanding of the alkaloid pathways, and genomic and EST sequencing in the alkaloid-producing species has become technically easier because of the advent of next-generation sequencing technology. These approaches should reveal potential candidate structural genes for the pathways. Bioinformatics analysis and detailed expression profiling are also promising strategies to select candidate genes from the sequence datasets. Nevertheless, in vitro and in vivo functions of each enzyme should be determined experimentally in subsequent analyses. Further elucidation of alkaloid biosynthesis will provide molecular tools for genetic manipulation of the pathways.

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# Chapter 17
# Polyamine Toxins from Spiders and Wasps

**Xiaofeng Xiong and Kristian Strømgaard**

**Abstract** Polyamine toxins, isolated from spider and wasp venoms, are a group of small molecular weight natural products with intriguing biological activities. They are secondary metabolites used for paralyzing prey, causing an immediate, but reversible, effect on the prey. Polyamine toxins have in particular been used as pharmacological tools for the study of ionotropic receptors, such as ionotropic glutamate (iGlu) receptors and nicotinic acetylcholine (nACh) receptors as nonselective open-channel blocker. Polyamine toxins have been used as templates to design novel molecular probes with improved properties over the naturally occurring compounds. In this chapter, we focus on the isolation and characterization of the polyamine toxins, as well as the recently developed synthetic strategies for the preparation of polyamine toxins and analogues. We also include the recent structure–activity relationship studies and application of polyamine toxins as biological tools, including developing selective iGlu subtype receptors and nACh receptors antagonists, as well as templates to develop labeled and fluorescent polyamine toxin probes.

**Keywords** Ionotropic glutamate receptors • Natural products • Nicotinic acetylcholine receptors • Polyamine toxin • Solid-phase synthesis • Spider and wasp

## 17.1 Introduction

Polyamine toxins are a group of secondary metabolites isolated from spiders and wasps (Olsen et al. 2011) that generally have molecular weights less than 1 kDa and are used for paralyzing preys, causing an immediate but reversible effect (Mueller et al. 1995). It was generally believed that most neurotoxins present in spiders and wasp venoms were high molecular weight compounds, such as larger peptides or proteins akin to the proteinaceous toxins from snake venoms. However, studies of polyamine toxins from spiders and wasps changed this belief as these natural products are indeed small molecules with many biological activities, including highly

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potent antagonists of neurotransmitter receptors, in particular the ionotropic glutamate (iGlu) receptors in invertebrate neuromuscular synapses (Usherwood et al. 1984; Eldefrawi et al. 1988; Piek et al. 1988). Kawai and colleagues were among the first to examine the physiological action of polyamine toxins and demonstrated that acylpolyamine fractions from the venom of the spider *Nephila clavata* (Joro spider) selectively blocked postsynaptic iGlu receptors in invertebrate neuromuscular synapses (Kawai et al. 1982, 1983; Abe et al. 1983). The active fractions from *Nephila clavata* and *Nephila maculate* spiders were named Joro spider toxins, such as JSTX-1, JSTX-2, JSTX-3, and JSTX-4 and nephila spider toxins such as NSTX-1, NSTX-2, NSTX-3, and NSTX-4 (Saito et al. 1985; Akaike et al. 1987; Priestley et al. 1989). At the same time, Usherwood and Usmanov respectively discovered that *Argiope* venom could block invertebrate and vertebrate glutamate receptors, which was followed by examination of a similar block of mammalian iGlu receptors (Kawai et al. 1982; Usherwood et al. 1984; Bateman et al. 1985; Usherwood and Duce 1985).

## 17.2 Isolation and Structure of Polyamine Toxins

In 1986, Volkova and colleagues performed the first full structural characterization of a polyamine toxin from the venom of the orb-weaver spider *Argiope lobata*, designated argiotoxin-636 (**1**, ArgTX-636; Fig. 17.1), where the number refers to the molecular weight of the compound (Schäfer et al. 1994). Soon after, a series of structurally related toxins was identified from the venom of *Argiope lobata* by different research groups (Adams et al. 1987; Grishin et al. 1989). These compounds shared several structural features, as they contain an arylacetyl moiety head group, which is connected to a polyamine unit by an asparagine residue or lysine in some cases, and generally an arginine amino acid tail (compounds **1**–**5**; Fig. 17.1), except for the truncated analogue (**6**, ArgTX-373; Fig. 17.1).

The first structural assignment of polyamine toxin from the venom of *Nephila clavata*, JSTX-3 (**7;** Fig. 17.1), was published by Nakajima and coworkers (Aramaki et al. 1987a, b). In addition, a large number of toxins were subsequently characterized from the venoms of *Nephila clavata*, *Nephila maculate*, *Nephila clavipes*, *Nephilengys borbonica*, and *Nephilengys cruentata*, which were named JSTXs, NSTXs, and NPTXs, respectively (Aramaki et al. 1987a, b; Teshima et al. 1987; Hashimoto et al. 1987; Chiba et al. 1994; Hisada et al. 1998; Palma and Nakajima 2005), with the numbers referring to the elution order in high performance liquid chromatography. Later, the common structural features of those toxins led to a division into four structural elements, where the aromatic acetyl group and polyamine backbone are key components and the amino acid linker and amino acid tail are optional moieties (Olsen et al. 2011; Hisada et al. 1998; Palma et al. 1998).

Another group of polyamine toxins isolated from funnel web spiders were isolated and characterized (compounds **11–14**; Fig. 17.1) in a similar manner. These toxins are also named according to the molecular mass, such as the α-agatoxin with a molecular mass of 489 Da, named AG489 or Agel-489 (Skinner et al. 1989;

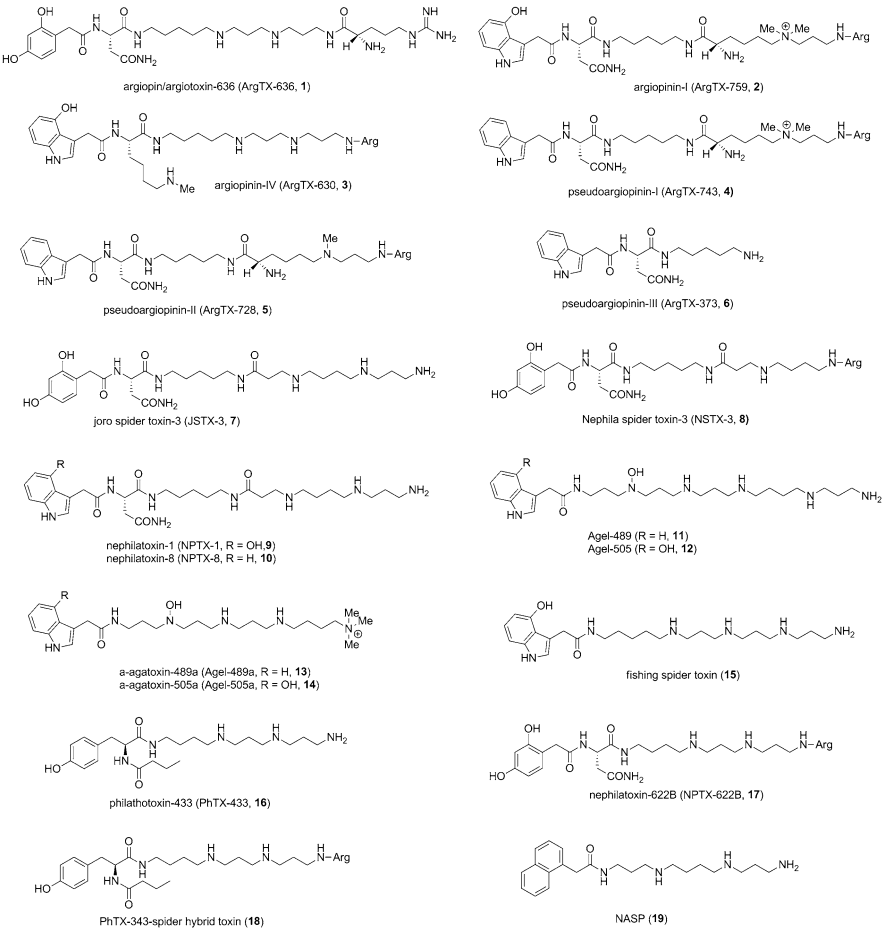



**Fig. 17.1** Structures of representative polyamine toxins

Adam et al. 1989; Jasys et al. 1990; Quistad et al. 1990). Shortly after, similar polyamine toxins were isolated from the venom of another funnel web spider, *Hololena cutra*, as well as *Agelenopsis aperta* (Jasys et al. 1992). Although toxins isolated from *Hololena cutra* have structures similar to both argiotoxins and nephila toxins, they differ in the nature and presence of amino acids, and importantly some of these have *N*-hydroxylated amino groups in the polyamine backbone. Polyamine toxins have also been isolated from different family of spiders, such as fishing spider toxin (**15**; Fig. 17.1), which was isolated from *Dolomedes okefinokensis* and shown to reversibly block the voltage-gated calcium channels (McCormick et al. 1993).

Philanthotoxin-433 (**16**, PhTX-433; Fig. 17.1) is a structurally related, albeit somewhat simpler polyamine toxin, which was isolated from the Egyptian digger wasp *Philanthus triangulum*. It was isolated, characterized, and synthesized by a collaborative effort by Nakanishi, Usherwood, and coworkers (Eldefrawi et al. 1988).

Among all the polyamine toxins, PhTX-433 has been most extensively used as template for structure–activity relationship (SAR) studies, pioneered by the work of Nakanishi and coworkers, as described later (Strømgaard et al. 2005; Andersen et al. 2006; Olsen et al. 2011). More recently, the structure of PhTX-433 and spider polyamine toxin NPTX-622B (**17**; Fig. 17.1) was combined in a spider–wasp hybrid analogue (**18**; Fig. 17.1) (Olsen et al. 2007).

In general, a large number of polyamine toxins from the venoms of spiders or wasp have been isolated and characterized, and a number of subsequent studies have revealed remarkable biological activities. One of the most characteristic properties of polyamine toxins is their ability to block ionotropic (iGlu) glutamate receptors. The iGlu receptors are important receptors in the mammalian central nervous system (CNS) and mediate the majority of excitatory synaptic transmission. Thus, iGlu receptors are crucial for normal brain function and are believed to be involved in numerous neurological diseases including Alzheimer's disease, brain damage following ischemia, and schizophrenia (Traynelis et al. 2010). The iGlu receptors are formed by a homomeric or heteromeric assembly of four subunits (Fig. 17.2), which form a cation channel that conducts $Na^+$, $K^+$, and $Ca^{2+}$ across the cell membrane. This action is controlled by the binding of glutamate to sites on the extracellular domain of iGlu receptors (Traynelis et al. 2010), which are divided into three main subtypes: *N*-metyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and kainate receptors.

Polyamine toxins are so-called noncompetitive open ion channel blockers of the iGlu receptors (Strømgaard and Mellor 2004; Mellor and Usherwood 2004; Strømgaard et al. 2005), where they are believed to bind to the central ion channel

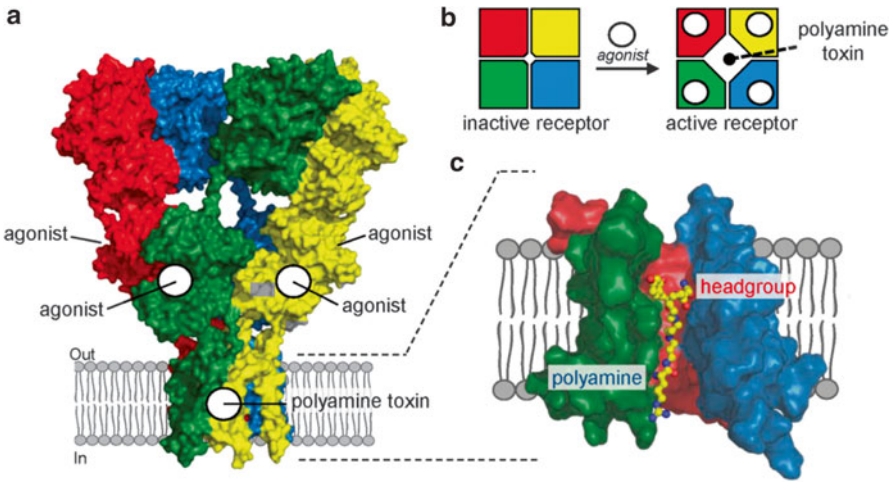


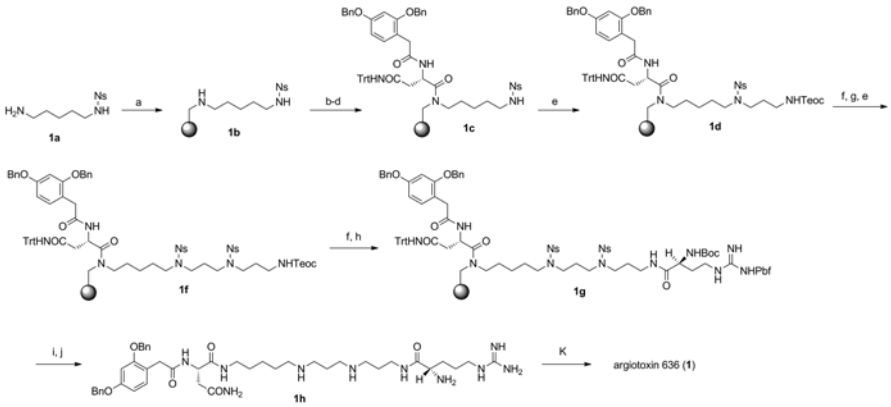

**Fig. 17.2** **a** X-ray crystal structure of homomeric GluA2 AMPA receptor (PDB ID 3KG2) with agonist and polyamine toxin binding sites indicated. **b** *Top view* of receptor showing opening of channel pore by agonist activation and subsequent pore blocking by polyamine toxins. **c** Modeling of polyamine toxin binding in the ion channel, showing the head group positioned in the vestibule and the polyamine moiety penetrating the narrow region of the ion channel (Nørager et al. 2013)

region and inhibit ion flow (Fig. 17.2). Interestingly, one of the only very few drugs that acting on iGlu receptors, memantine (Ebixa), which is used in the symptomatic treatment of Alzheimer's diseases, employs a similar mode of action (Lipton 2007; Cosman et al. 2007). However, what makes polyamine toxins unique pharmacological tools is their ability to distinguish among iGlu receptors that are $Ca^{2+}$ permeable and those which are not: $Ca^{2+}$ permeability in AMPA receptors is, generally speaking, regulated by a single amino acid in the so-called Q/R site, where an asparagine (Q) renders the receptor $Ca^{2+}$ permeable, whereas an arginine (R) in the same position does not (Poulsen et al. 2014). In addition to their action at iGlu receptors, polyamine toxins have also displayed activity at other ionotropic receptors, particularly the nicotinic acetylcholine (nACh) receptors. Their interesting biological properties mean that polyamine toxins are useful tools in investigations of both invertebrate and vertebrate nervous systems in addition to serving as lead structure for the design and synthesis of potential therapeutic agents targeting diseases in the brain.

## 17.3 Chemical Synthesis of Spider and Wasp Polyamine Toxins

Polyamine toxins have been synthesized using conventional solution-phase strategies since the late 1980s, when the structures were first revealed (Kuksa et al. 2000; Karigiannis and Papaioannou 2000). In the polyamine toxins, a solution-phase strategy can be less attractive because of to the extensive use of protecting groups, including several protection and deprotection steps, as well as workup and purification of highly polar polyamine intermediates. Therefore, there was an interest in developing synthetic methods based on solid-phase synthesis (SPS), and the first such strategies were reported in 1994 for the synthesis of the structurally simple NPTX-9 and NPTX-11 (Bycroft et al. 1994). Subsequently, a large number of studies have examined SPS for the synthesis of polyamine toxins (Strømgaard et al. 2001; Strømgaard and Mellor 2004; Olsen et al. 2005). Although SPS is generally more expensive than solution-phase synthesis, SPS has a number of advantages: Purification of polar intermediates has been reduced to simply washings, a range of different protecting groups are easily employed, and parallel synthesis of polyamine toxin analogues for SAR studies is easier (Strømgaard et al. 2001). The first total synthesis of a spider polyamine toxin, which included constructing the polyamine moiety on a solid support, was achieved by Wang and coworkers (Wang et al. 2002): by applying a borane reduction of a trityl resin-bound tripeptide as a key step, they prepared Agel-416b in 37 % overall yield.

Bidirectional SPS, which enables the independent prolongation or derivatization of a linear polyamine at two terminal points, was developed by Bienz and coworkers (Manov and Bienz 2001). By anchoring the monoprotected amine to the resin and alkylated the resin-bound secondary amine, followed by selective deprotection of either of the two terminal amino functions, a large number of polyamine derivatives were easily constructed (Manov and Bienz 2001). Recently, a total SPS of ArgTX-636 and its analogues using a backbone amide linker (BAL) resin enabled




**Scheme 17.1** Bidirectional solid-phase total synthesis of ArgTX-636. The monoprotected diamine was anchored to the resin by reduced amination, followed by adding the amino acid linker and aromatic head group under the standard coupling condition. The polyamine backbone was prolonged by repeated Fukuyama–Mitsunobu reactions. After adding the terminal arginine to the intermediate, the protected resin-bound molecule was released from the resin, and then the protecting group was removed to obtain the nature ArgTX-636

the bidirectional functionalization of polyamine toxins (Nelson et al. 2009) (Scheme 17.1). Briefly, this was achieved by anchoring a monoprotected diamine to the resin, followed by two amino acid coupling reactions. The polyamine moiety was then generated by a Fukuyama–Mitsunobu alkylation strategy (Vigil-Cruz and Aldrich 1999) with amino alcohols as building blocks. This step generated fully protected, resin-bound ArgTX-636, and subsequent deprotection and cleavage from the resin provided the natural product ArgTX-636 (Nelson et al. 2009). The methodology was used for the synthesis of systematically modified ArgTX-636 analogues (Nelson et al. 2009; Poulsen et al. 2013) and later a similar strategy was applied for the synthesis of Joro spider Toxin-4 (Barslund et al. 2010), NPTX-1, and NPTX-8 (Lucas et al. 2012). Very recently, *N*-methylated and *N*-hydroxylated spider polyamine toxins were also successfully prepared on SPS by utilizing a similar strategy combined with selective *N*-methylation and *N*-hydroxylation of secondary amines in the polyamine moiety; *N*-methylated argiopinines and pseudoargiopinines I and II, as well as *N*-hydroxylated Agel-489 and Agel-505, were also obtained (Nørager et al. 2014).

## 17.4 Polyamine Toxins as Biological Tools

### *17.4.1 Development of Selective iGlu Receptor Inhibitors*

Polyamine toxins have been used as pharmacological tools for neurobiological studies, particular those related to iGlu receptors (Jackson and Usherwood 1988; Jackson and Parks 1989; Kawai et al. 1991). Early studies revealed that polyamine

toxins had very different activities among subtypes of iGlu receptors in both vertebrate and invertebrate systems, which raised the possibility of identifying subtype-selective antagonists for iGlu receptors. It was later found that polyamine toxins are so-called open-channel blockers, which means that they only block the open state of the iGlu, when glutamate is bound, which is also indicative of being use dependent. Tikhonov and coworkers have used computational approaches to generate models for how spider polyamine toxins and polyamine ion channel blockers work (Tikhonov 2007). These models suggested that the toxins permeate the ion channel domain, and the polyamine tail penetrates through the narrowest part of the channel, the selectivity filter, whereas the aromatic head group is located in the area just above the selectivity filter. The cell membrane potential is important for the interaction between polyamine toxins and iGlu receptors, and the potency increases, and toxin dissociation slows, with increasing the negative membrane potential; thus, polyamine toxins are generally highly voltage-dependent blockers of iGlu receptors. As mentioned previously, iGlu receptors are divided into NMDA, AMPA, and kainate receptor subtypes, and it has been recognized that polyamine toxins have different affinities for these iGlu subfamilies (Priestley et al. 1989; Draguhn et al. 1991; Mueller et al. 1991; Parks et al. 1991). For AMPA and kainite receptors, polyamine toxins, such as JSTX-3 and PhTX-433, as well as the synthetic analogue, 1-naphthylacetyl spermine (**19**, NASP; Fig. 17.1) (Koike et al. 1997), have become an important tools for studying $Ca^{2+}$-permeable AMPA receptors because of their unique property of only inhibiting this subgroup of AMPA receptors.

Recent work has focused on developing iGlu subtype-selective polyamine toxins through SAR studies. In 2009, the synthesis and a SAR study of ArgTX-636 and analogues were performed (Nelson et al. 2009). Taking advantage of the developed SPS methodology, ArgTX-636 and eight close analogues were prepared by introducing subtle changes in the polyamine moiety, as well as in the guanidino group of the Arg moiety. The compounds were tested for inhibitory activity at AMPA and NMDA receptor subtypes, showing that the guanidino group is important for iGlu receptor activity. On the other hand, replacing either one of the secondary amino groups in the polyamine moiety, analogues **20** and **21** (Fig. 17.3), led to remarkable shift the selectivity, with **20** and **21** being potent and selective inhibitors of AMPA and NMDA receptors, respectively. Recently, a more comprehensive SAR study of ArgTX-636 was carried out (Poulsen et al. 2013), wherein the importance of secondary amines of the polyamine moiety, the head group and linker amino acid, were systematically evaluated for activity at AMPA and NMDA receptors, revealing subtle correlation between locations of the secondary amino groups and inhibition of either AMPA or NMDA receptors. Moreover, changes in the head group and linker amino acid led to two analogues potent and selective to NMDA and AMPA receptors, compounds **22** and **23**, respectively (Fig. 17.3). In another SAR study of *N*-methylated argiopinines, pseudoargiopinines I and II, and *N*-hydroxylated Agel-489 and Agel-505, it was observed that *N*-methylations of polyamine toxins generally do not affect selectivity between AMPA and NMDA receptors while the inhibitory potency was gradually reduced upon *N*-methylation (Nørager et al. 2014).

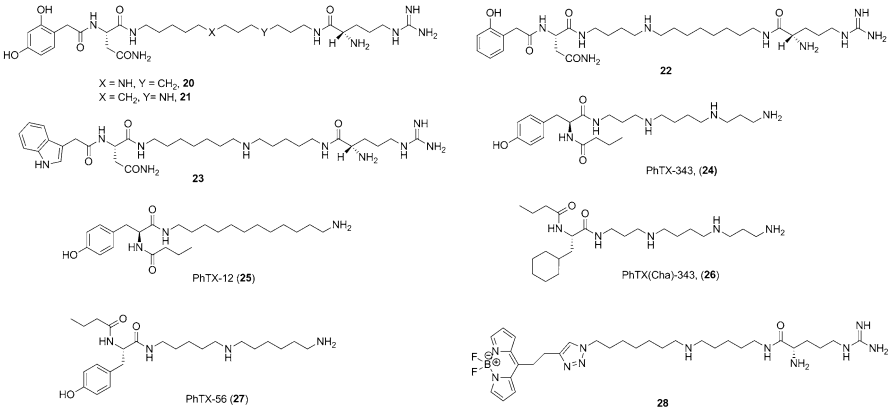



**Fig. 17.3** Polyamine toxins analogues with improved properties compared to their native counterparts, as well as a fluorescent polyamine toxin analogue

In addition to the recent studies of spider polyamine toxins, the wasp toxin PhTX-433 has also been used as a template in attempts to develop more potent and selective compounds. As described next in relationship to nACh receptors, Nakanishi and colleagues have contributed significantly to this area. Recently, a number of PhTX-433 have been designed, synthesized, and evaluated for inhibitory potency, in particular at AMPA receptors, leading in several cases to fairly potent and AMPA receptor selective analogues (Frølund et al. 2010; Kromann et al. 2002).

### *17.4.2 Development of Selective nACh Receptor Ligands*

Among native polyamine toxins, PhTX-433 is the best characterized antagonist of nACh receptors. PhTX-433 and its synthetic analogue PhTX-343 (**24**; Fig. 17.3), with a symmetrical polyamine moiety, have been used as lead structures in SAR studies at nACh receptors. This development was pioneered by Nakanishi and coworkers, who prepared more than 100 PhTX-433 analogues and tested them for pharmacological activity (Nakanishi et al. 1994). In a systematic study of PhTX-343, replacement of the secondary amino groups with methylene groups or oxygen atoms dramatically increased the inhibition of nACh receptors, while reducing potency at iGlu receptors, leading to the identification of PhTX-12 (**25**; Fig. 17.3) (Strømgaard et al. 1999), which provided the first example of a polyamine toxin analogue that could discriminate between nACh receptors and iGlu receptors (Mellor et al. 2003; Strømgaard et al. 1999). Later studies showed that the when the number of methylene groups between primary amino group and the aromatic head

group of PhTX-12 was less than 11, there was a significant decrease in antagonism at nACh receptors (Strømgaard et al. 2002). Similarly, a marked decrease in activity was found when replacing the *N*-butanoyl group of PhTX-12 with more hydrophobic groups (Strømgaard et al. 2000).

In 2006, Olsen and coworkers prepared a family of cyclohexylalanine-containing analogues of PhTX-343 (Olsen et al. 2006). One of the compounds, PhTX(Cha)-343 (**26**; Fig. 17.3) was 277 fold more potent than PhTX-343 at muscle-type nACh receptors, thus being one of the most potent polyamine toxin-based inhibitors of this receptor. The inhibition by PhTX(Cha)-343 is voltage dependent and is thus believed to block the open-channel state of the receptor (Olsen et al. 2006).

### *17.4.3 Development of Labeled and Fluorescent Polyamine Probes*

In response to their interesting biological properties, there has been interest in developing polyamine toxins wherein various forms of labels, such as radioactive or fluorescent, are introduced into the compound without compromising the biological activity. Early examples include the iodine-labeled JSTX-3 (Hagiwara et al. 1988; Shimazaki et al. 1988) and philanthotoxins (Goodnow et al. 1991), which were developed as molecular probes to study both iGlu and nACh receptors. In 2005, Strømgaard and coworkers prepared a range of labeled analogues of PhTX-56 (**27**; Fig. 17.3), which allowed for molecular level studies of ligand–receptor interaction, as well as other studies pertaining to AMPA receptor structure and function (Andersen et al. 2005). Apart from that, philanthotoxin analogues containing photolabile moieties for receptor crosslinking were prepared for studying the inhibitory mechanism and binding to ion channels of nACh receptors (Choi et al. 1995; Fang et al. 1998).

More recently, polyamine toxins comprising fluorescent moieties have been developed. Such compounds have exciting prospects, as they potentially can be used to uniquely label $Ca^{2+}$-permeable AMPA receptors in neurons and neuronal tissues. First, fluorescently labeled probes based on NPTX-594 were prepared by incorporating the fluorescent hydroxyl-coumarin structure, and later used in an assay related to paralysis of crickets (Nishimaru et al. 2009). Very recently, Strømgaard and coworkers designed and synthesized a set of fluorescent analogues using ArgTX-636 and in particular the selective argiotoxin analogues as templates (Nørager et al. 2013). By utilizing a revised SPS procedure, including on- and off-labeling strategies, 18 fluorescent polyamine toxin analogues were prepared. Subsequent biological evaluation on iGlu receptors showed that one compound (**28**; Fig. 17.3) had an $IC_{50}$ value of 11 and 86 nM on AMPA and NMDA receptors, respectively. Subsequently, one of the fluorescent polyamine toxins was used to visualize NMDA receptors in hippocampal neurons (Nørager et al. 2013).

## 17.5 Summary and Outlook

Although many polyamine toxins have already been isolated, there are strong implications that many more are awaiting discovery. Mass spectroscopic methodologies have proven particularly useful for the detection and characterization of new polyamine toxins from spiders, which have also allowed a scheme for classification of different polyamine toxins based on their chemical structure. The development of SPS strategies for preparing native polyamine toxins, and especially a range of analogues with increasing structural diversity, promises that many such new toxins can be readily prepared and employed in biological studies. Developments in these two areas could provide substantial new discoveries of the chemistry of polyamine toxins.

One of the major drivers of studies employing polyamine toxins is their interesting biological properties. Hitherto, polyamine toxins were successfully used as lead compounds to develop analogues with improved potency and selectivity toward iGlu and nACh receptors. Further developments could include design, synthesis, and application of labeled polyamine toxins as molecular probes to reveal the properties of a particular iGlu receptor in neurons.

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# Part VIII
# Action of Polyamines on Ion Channels

# Chapter 18
# Polyamine Block of Inwardly Rectifying Potassium (Kir) Channels

**Harley T. Kurata**

**Abstract** Inwardly rectifying potassium (Kir) channels were first identified based on their unique functional property of preferential conductance of potassium ions into the cell. The property of inward rectification differs significantly from their more widely studied Kv (voltage-gated potassium) channel relatives, which exhibit outward rectification resulting from voltage-dependent opening and closing of the channel pore. Rather, inward rectification arises from asymmetrical voltage-dependent blockade of these channels by endogenous intracellular polyamines. This distinct role of polyamines enables the physiological function of Kir channels to maintain a substantial potassium conductance when cells are at rest, but to shut down their conductance when faced with depolarizing stimuli to allow excitation events (e.g., action potentials) to take place. Functional studies of cloned Kir channels, and recent crystallographic insights, have revealed the importance of numerous side chains that line the channel and interact with polyamines as they move toward a stable binding site. The displacement of permeating $K^+$ ions in the channel pore, coupled to polyamine migration through the pore, underlies the very steeply voltage-dependent blockade.

**Keywords** Andersen's syndrome • Electrophysiology • Inward rectifier • Ion channel • Long QT • Polyamine • Potassium channel • Short QT • Spermine

## 18.1 Introduction

Inwardly rectifying potassium (Kir) currents were first characterized decades ago in skeletal and cardiac muscle fibers. Also initially referred to as 'anomalous' rectifiers, these currents were named for their unique characteristic of preferentially conducting inward currents, while outward currents were inhibited (Hutter and Noble 1960; Noble 1962). This trait contrasted with the outward rectification and intrinsic voltage dependence of the classic voltage-dependent $K^+$ (Kv) currents in excitable

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cells first described by Hodgkin and Huxley (Hodgkin and Huxley 1952). The first inwardly rectifying potassium (Kir) channel genes were cloned in the early 1990s, with reports of cloning IRK1 (now referred to as Kir2.1 or KCNJ2) (Kubo et al. 1993a), GIRK1 (now Kir3.1 or KCNJ3) (Kubo et al. 1993b), and ROMK1 (now Kir1.1, or KCNJ1) (Ho et al. 1993). These major steps forward allowed for heterologous expression of these ion channel genes, comparison of their functional properties, and identification of the major sequence determinants of Kir channel function.

Although many chapters in this textbook are concerned with important roles of polyamines in cell growth, metabolism, and proliferation, it turns out that polyamines also play a major role in cellular electrical behavior by controlling inward rectification of Kir channels. The identification of polyamine block as the underlying physiological mechanism of inward rectification was hinted at by a few early publications describing that rectification was not an 'intrinsic' property of the inward rectifying conductance (Vandenberg 1987; Matsuda et al. 1987). That is, if channels were removed from the cellular environment (by excised patch-clamp recordings, or highly effective dialysis of intracellular contents), properties of inward rectification could be weakened or abolished. This was a particularly unique mechanism of ion channel regulation, because most voltage-dependent regulatory mechanisms were presumed to be caused by conformational changes of ion channel proteins themselves, rather than an extrinsic factor. Subsequent screening of active components in specific cell lysate fractions led to the identification of polyamines as the major determinant of steeply voltage-dependent inward rectification of cloned IRK1 (Kir2.1) channels (Lopatin et al. 1994). All polyamines are able to block Kir channels, although spermine and spermidine are typically the most potent, whereas shorter endogenous polyamines such as putrescine are weaker blockers. The properties of polyamine block match several important features of currents described in primary cell culture systems such as cardiomyocytes, notably the steep effective valence of blockade and the strong coupling of the voltage dependence of rectification with the reversal potential of $K^+$ ions (Lopatin and Nichols 1996). For the remainder of this chapter, inward rectification is primarily discussed in the context of Kir2.1 channels, which are the most widely studied model of polyamine block, and also exhibit the most potent and steeply voltage-dependent polyamine block (Nichols and Lopatin 1997).

## 18.2 Characteristics of Polyamine Block

### *18.2.1 Steepness of Voltage-Dependent Block*

To an electrophysiologist, one of the most striking traits of polyamine block is the very high 'effective valence' of the blocking process. Voltage-dependent blockers such as polyamines are unique because blocker affinity for the ion channel target is not fixed, but rather affinity varies with changes in transmembrane voltage. A common way to assess this feature experimentally is to record ionic currents in

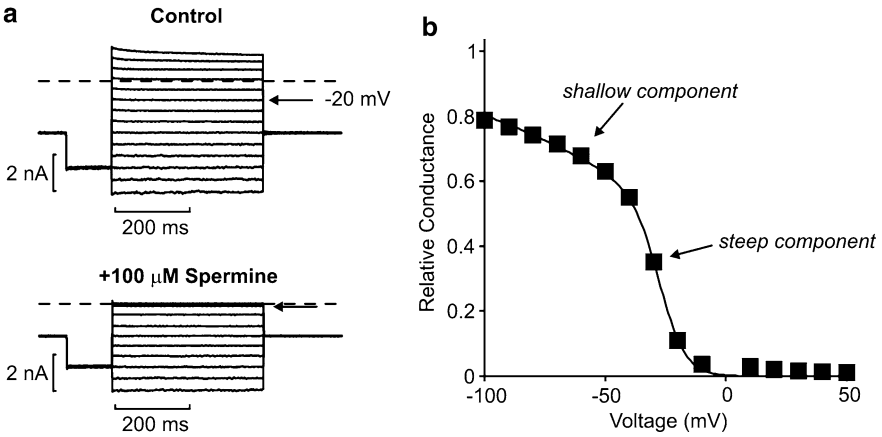


**Fig. 18.1** Voltage-dependent block of Kir2.1 channels by spermine. **a** Currents recorded using the inside-out patch-clamp method, in symmetrical 150 mM $K^+$ solutions. Membrane patches containing Kir2.1 channels were stepped between −100 and +30 mV in 10-mV steps, in the presence and absence of 100 **μ**M spermine. Note the large outward currents (above the *dashed* 'zero current' *line*) in control conditions, but strong inhibition of outward currents by spermine. **b** Relative conductance is calculated by dividing the current magnitude in spermine vs control at each voltage. At negative voltages, spermine effects are very weak, whereas at positive voltages currents are strongly inhibited

the absence and presence of a known blocker concentration, at different membrane voltages (see Fig. 18.1a). In the absence of polyamines or divalent ions such as $Mg^{2+}$, Kir channels behave as ohmic resistors over a physiological range of membrane voltage (Guo and Lu 2002). However, in the presence of polyamines, current inhibition becomes very significant at depolarized voltages. The percentage of current inhibition can be calculated at each voltage, and plotted graphically to illustrate the voltage dependence of the blockade (Fig. 18.1b). In the simplest scenario of a two-state equilibrium (open ↔ blocked), voltage-dependent blockade (i.e., the fraction of control current that remains in the presence of the blocker) is sigmoidal and can be described by a Boltzmann function such as

$$\frac{I}{I(\text{control})} = 1 \Big/ \left( 1 + \frac{[\text{spermine}]}{Kd(0\ \text{mV}) * e^{-\left(\frac{z\delta FV}{RT}\right)}} \right)$$

In this equation, $Kd_{(0\ \text{mV})}$ reflects blocker affinity for the channel in the absence of any membrane potential, and $z\delta$ describes how quickly blocker affinity changes with membrane voltage: larger $z\delta$ values reflect a steeper change of blocker affinity as membrane voltage is changed. Typically, $z\delta$ is interpreted as being the product of the valence of the blocker (i.e., +4 for spermine), and the fraction of the transmembrane field traversed by the blocker en route to its final binding site (i.e., 0 if the blocker does not enter the transmembrane field, or 1 if the blocker completely

traverses the transmembrane field, so $0<\delta<1$). In reality, the voltage dependence of polyamine block of Kir2.1 channels is not quite this simple, and generally exhibits multiple components (a shallow and a steep component; Fig. 18.1b), corresponding to distinct low- and high-affinity binding sites (Xie et al. 2002).

Most notably, the voltage dependence of polyamine blockade is very steep relative to other commonly used ion channel blockers, meaning that in a physiological setting where polyamines are present, strongly rectifying Kir channels switch from being conductive to strongly inhibited over a very narrow voltage range. Moreover, a notable feature of polyamine block of Kir channels is that the effective valence of spermine block is higher than the charge of the blocker. Specifically, the effective valence of spermine and spermidine block of Kir2.1 channels is typically reported to be nearly 5 (Guo and Lu 2003). Because the charge of spermine can reach a maximum of +4, this property cannot be rationalized solely on the basis of migration of the charged blocker across the transmembrane voltage difference (Woodhull 1973).

### *18.2.2 Coupling of Polyamine Blockers and Permeating Ions*

The second classical feature of inwardly rectifying potassium channels is that the degree of rectification depends significantly on the reversal potential of $K^+$, and particularly the extracellular $K^+$ concentration (Hagiwara et al. 1976). Specifically, as the extracellular $K^+$ concentration is increased with a constant intracellular $K^+$ concentration (thereby shifting the $K^+$ reversal potential to more positive voltages), the degree of rectification at a given voltage becomes less pronounced. In the simple descriptive equation for voltage-dependent block shown above, this phenomenon would be described as a $K^+$-dependent change in $Kd_{(0\,\mathrm{mV})}$. This permeant ion dependence of affinity is thought to reflect interactions between ions and blockers in the pore. An important but not always appreciated aspect of this process is the strong asymmetrical dependence on $K^+$ concentration. Extracellular $K^+$ has a far more dramatic impact on polyamine block than intracellular $K^+$, although there is not a well-accepted mechanistic explanation for this asymmetry (Lopatin and Nichols 1996; Kurata et al. 2010).

## 18.3 Inwardly Rectifying Potassium Channel Ontogeny and Structures

### *18.3.1 Kir Channel Genes*

Kir channels are categorized into seven families (Kir1–7) with a total of at least 15 different genes/subunits (in humans) (Kubo et al. 2005; Hibino et al. 2010). A functional Kir channel comprises four pore-forming subunits and can be homo- or heterotetrameric, with heteromerization permitted among members of the same family

(e.g., Kir2.1 with Kir2.2, Kir3.1 with Kir3.4) (Hibino et al. 2010), and in some cases with obligatory auxiliary subunits as with Kir6 channel assembly with sulfonylurea receptors (Inagaki et al. 1995). A peculiarity of the Kir channel family is that although they are collectively referred to as inward rectifiers, inward rectification is not especially prominent in many of these channel types. Kir2 family channels exhibit strong rectification and are the prototypical ion channels for the study of polyamine block. Kir3 and Kir4 family channels exhibit intermediate rectification properties (i.e., weaker affinity and shallower voltage dependence relate to Kir2 channels), whereas Kir1 and Kir6 family channels are extremely insensitive to polyamine block. As is discussed later, these differences in polyamine sensitivity can be attributed to a small subset of amino acids that differ between these Kir subfamilies (Hibino et al. 2010).

### *18.3.2 Structures and Domain Architecture of Kir Channels*

Recent atomic resolution structures of Kir channels have significantly enhanced our understanding of Kir channel function and provide an important framework to understanding their interactions with polyamines (Whorton and MacKinnon 2011; Hansen et al. 2011) (Fig. 18.2). The Kir channel transmembrane pore consists of two transmembrane α-helices from each subunit, linked via a re-entrant segment that forms a selectivity filter similar to virtually all $K^+$-selective channels.

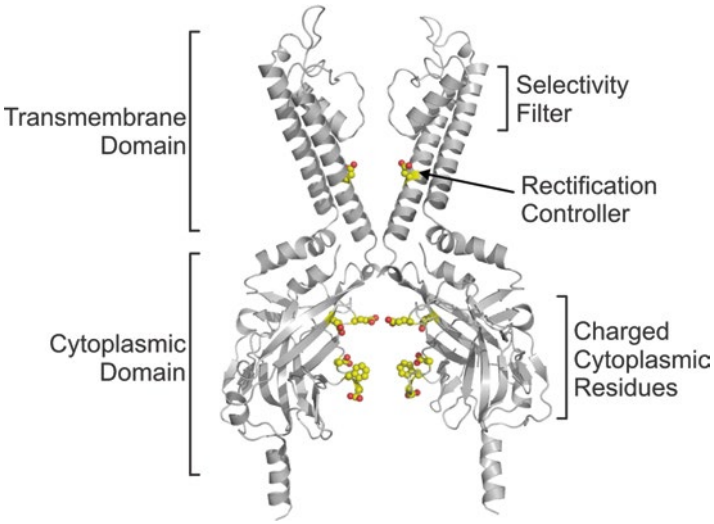


**Fig. 18.2** Atomic resolution structure of Kir2.2. Coordinates used were reported by Hansen et al., (2011). Two subunits have been removed to more clearly depict the central pore axis that forms the permeation pathway. The rectification controller residue, and cytoplasmic residues important for polyamine block, are highlighted as *spheres*

Importantly, these channels are not intrinsically sensitive to voltage because they lack the canonical voltage-sensing domain that endows other channel types with voltage sensitivity (Yellen 2002). The transmembrane helices of each subunit comprise the outer helix (TM1) and the inner helix (TM2) that lines the pore. Most crystal structures of Kir channels have been captured in the closed state, and no fully open conformation of a Kir channel has yet been reported, although some 'partially open' conformations of Kir2.2 and Kir3.2 have been described (Hansen et al. 2011; Whorton and MacKinnon 2011), along with a mutant KirBac3.1 channel designed to prop open the channel pore (Bavro et al. 2012). In all cases, crystallographic evidence indicates that Kir channel opening involves widening of an aperture in the bundle-crossing region of the channel, and this is consistent with several functional studies using accessibility of MTS reagents or polyamine blockers to study gating conformations of these channels (Phillips and Nichols 2003; Phillips et al. 2003). In addition to the canonical $K^+$ channel pore-forming domain, Kir channels comprise a large cytoplasmic domain. The transmembrane and cytoplasmic domains form a stable interface generating a single long ion-conducting pore. The cytoplasmic domain is composed of both the N- and C-termini of the protein and forms binding sites for numerous permeating ions, intracellular ligands, and polyamine blockers.

The additional $K^+$ ions housed in the extended Kir channel pore likely play a role in generating the steep voltage dependence of the block already discussed (Xu et al. 2009), because polyamine movement through the pore requires the displacement of ions that occupy binding sites ahead of the blocker (Fig. 18.3). That is, as polyamines migrate toward their stable binding site, ions lying ahead of the blocker in the pore are forced to move through the transmembrane field, and thereby affect the voltage dependence of the process. An understanding of the details of ion-binding sites and the mechanisms that couple blocker and ion movement is important to describe the steep voltage dependence of these channel types and the observed dependence on $K^+$ concentration gradients. Although a firm description of interactions between

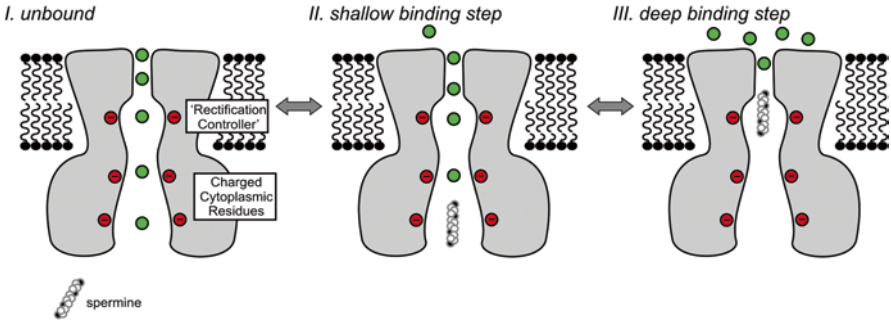


**Fig. 18.3** Coupled movement of blockers and ions in Kir channel pores. Each image is a schematic of a Kir channel, depicting the progressive movement of spermine as it interacts with the cytoplasmic domain of the channel and migrates toward its stable binding site. As the blocker moves toward the deep binding site, multiple ions are displaced through the transmembrane field, generating the steep voltage dependence associated with polyamine block

blockers and permeant ions remains elusive, crystal structures have also provided valuable insights into mapping specific ion-binding sites along the Kir channel pore. The selectivity filter of Kir channels closely resembles other $K^+$-selective channels and comprises four binding sites for $K^+$ ions formed by backbone carbonyl atoms. In addition, a commonly observed $K^+$ ion-binding site (the 'cavity site') is apparent in the inner cavity region of Kir channels. Additional binding sites that are unique to Kir channels are formed by the intracellular cytoplasmic domain, with up to five ions visible in some crystal structures (Pegan et al. 2005; Nishida et al. 2007; Xu et al. 2009). Overall, the large number of ion-binding sites that have been identified in Kir channel structures is consistent with the notion that a significant component of the voltage dependence of polyamine block arises from the displacement of ions ahead of the blocker, rather than movement of the charged blocker itself through the transmembrane field (Pearson and Nichols 1998; Guo and Lu 2003).

### *18.3.3 Important Residues for Polyamine Binding*

Kir channel proteins exhibit strong sequence conservation across all subfamilies but markedly different rectification properties and sensitivities to polyamine block (Hibino et al. 2010). For example, channels of the Kir2 subfamily exhibit a potent spermine block that is steeply voltage dependent, whereas Kir1 or Kir6 subfamily channels exhibit very weak inhibition by spermine or other polyamines. The sequence similarity between channels with significant functional differences was highly amenable to chimeric approaches, and rational site-directed mutagenesis, to identify key residues that control polyamine binding (Taglialatela et al. 1994; Wible et al. 1994; Kubo and Murata 2001; Lu 2004). These experiments have demonstrated that strong rectification in Kir2 channels can be attributed to rings of pore-lining acidic residues (generally considered to carry negative charges) in both the cytoplasmic and transmembrane domains of the channel.

#### 18.3.3.1 Inner Cavity Residues That Control Polyamine Block

The most important determinant of polyamine sensitivity of Kir channels lies within the pore-lining TM2 helix, at Kir2.1 residue D172 (Wible et al. 1994; Lopatin et al. 1994) (Fig. 18.2). This residue is frequently referred to as the 'rectification controller.' Neutralization of this residue in polyamine-sensitive channels such as Kir2.1 significantly weakens polyamine block. Consistent with this finding, introduction of acidic residues into polyamine-insensitive channels such as Kir1.1 or Kir6.2 endows strong sensitivity to polyamine block, with steep voltage dependence (Lu and MacKinnon 1994; Shyng et al. 1997). It is noteworthy that there is not a strict positional requirement for a TM2 negative charge to introduce polyamine sensitivity: substitution of a negative charge at any pore-lining position in the transmembrane domain of Kir6.2 has been shown to introduce strong polyamine sensitivity

(Kurata et al. 2004). However, among naturally occurring Kir channels, negatively charged residues are virtually never present at any position except the rectification controller D172 equivalent.

#### 18.3.3.2 Cytoplasmic Domain Residues That Control Polyamine Block

There are also numerous pore-lining acidic residues in the cytoplasmic domain (Kir2.1 residues E224, D255, D259, E299), along with a pore-lining aromatic residue (Kir2.1 residue F254) that primarily control low-affinity interactions with polyamines and also alter inward rectification properties when mutated (Fig. 18.2). Broadly speaking, mutations at these positions tend to impact the kinetics of polyamine block/unblock, and also the presence of the shallow voltage-dependent component of polyamine block (Shin et al. 2005; Kurata et al. 2007) (see also Fig. 18.1). However, mutations at E224 or E299 also diminish the overall voltage dependence and affinity of polyamine block (Kubo and Murata 2001; Guo and Lu 2003). More specifically, polyamine interactions with residues in the cytoplasmic domain are thought to underlie the early rate-limiting step of polyamine block, which is followed sequentially by polyamine migration into the inner cavity. This second step (entry of polyamines into the inner cavity) is associated with displacement of significantly more charge, and is thus far more steeply voltage dependent than the weak association of polyamines with the cytoplasmic domain (Fig. 18.3) (Shin and Lu 2005; Kurata et al. 2007). However, binding of polyamines in the cytoplasmic domain has been suggested to facilitate blocker entry into the inner cavity by increasing the local polyamine concentration (Lopatin et al. 1995; Kubo and Murata 2001; Xie et al. 2002).

### *18.3.4 The Nature of the Terminal Polyamine-Binding Site*

Among the most debated aspects of polyamine block of Kir channels has been the details of the 'stable' polyamine-binding site in the Kir channel inner cavity. Although the role of the rectification controller residue is not in question, some studies have argued for a polyamine orientation with the leading end of the blocker located in the vicinity of the rectification controller (Guo and Lu 2003; Lu 2004), whereas others have argued for a deeper binding site between the rectification controller and the selectivity filter (Chang et al. 2003; John et al. 2004; Kurata et al. 2006, 2010). Overall, the first hypothesis is motivated primarily by data demonstrating that residues within the cytoplasmic domain may have significant effects on the stability of the 'deep' binding equilibrium for polyamines, suggesting that they contribute to the polyamine-binding site in some way. However, a deeper binding site is indicated by data demonstrating that polyamines can be trapped in the inner cavity after the introduction of positively charged adducts (such as MTSEA or MTSET) near the cytoplasmic entrance of the inner cavity. These data suggest that spermine

likely binds above the introduced adducts just one α-helical turn away from the rectification controller (toward the cytoplasmic side, at Kir2.1 position I176C, or Kir6.2 position L164C) (Kurata et al. 2010, 2013). Missing from the dialogue on polyamine binding is a crystal structure convincingly demonstrating the orientation of polyamines in the inner cavity deep binding site. Future experiments will surely continue to refine descriptions of polyamine binding.

## 18.4 Diseases Related to Polyamine-Sensitive Kir Channels

The Kir2 family are the most sensitive Kir channels to voltage-dependent polyamine block. Their physiological roles are best understood in excitable tissues such as skeletal and cardiac muscle where they maintain resting membrane potential and shut off upon depolarization to allow an action potential. Not surprisingly, loss- and gain-of-function mutations of Kir2 family channels have interesting consequences on cardiac function, and other consequences related to bone and muscle development also emerge as a result of these channel defects. Loss of function mutations in Kir2.1 result in Andersen's syndrome, an autosomal dominant genetic disorder characterized by cardiac arrhythmias caused by prolongation of the action potential (long QT syndrome, LQT7), periodic muscle paralysis, and abnormal facial and digital bone structures (Plaster et al. 2001; Tristani-Firouzi and Etheridge 2010). Many associated mutations either disrupt channel interactions with $PIP_2$ or impair channel trafficking (Tristani-Firouzi et al. 2002; Lopes et al. 2002).

The functional counterpoint to Kir2.1 loss-of-function is mutations that cause gain-of-function. Remarkably, mutations of Kir2.1 residue D172 that disrupt polyamine binding have been reported to cause a form of short QT syndrome (SQT3) (Priori et al. 2005). This defect results in shortening of the cardiac action potential and is also associated with arrhythmias and cardiac death. The first SQT3 mutation reported is in fact the D172N mutation that has been commonly used to study polyamine block of Kir2.1 channels, corresponding to neutralization of the rectification controller. Given the large number of residues identified as important for polyamine block (see Sect. 18.3.3), it seems likely that other Kir2 channel mutations will emerge in studies of SQT3; however, at present no additional mutations have been reported.

## 18.5 Summary

Polyamine block of Kir channels is an essential physiological mechanism of ion channel regulation that is distinct from the more commonly studied roles of polyamines in cell growth and proliferation. Intracellular polyamines enter the Kir channel pore at depolarized voltages, causing preferential blockade of outward $K^+$ currents and allowing cellular electrical excitation to proceed. Recently identified mutations of the Kir2.1 channel cause disruption of polyamine block and are linked to cardiac arrhythmias.

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# Chapter 19
# Polyamine Action on Plant Ion Channels and Pumps

**Igor Pottosin**

**Abstract** Polyamines (PAs) regulate growth and stress responses in plants. Among the vitally important roles of PAs is a modulation of ion transport across vacuolar and plasma membranes. PAs at micromolar concentrations block two major vacuolar cation channels, namely, the slow (SV = TPC1, tandem-pore calcium channel) and the fast (FV) activating ones. These effects are direct and fully reversible, with a potency descending in a sequence Spm > Spd > Put. However, effects of polyamines on the plasma membrane cation and $K^+$-selective channels are hardly dependent on the PA species, display a relatively low affinity, and are indirect. Plants widely implement a mechanism, including the PAs export to the apoplast and catabolization therein, resulting in a generation of reactive oxygen species (ROS). ROS in turn activate a variety of ion conductances, underlying $Ca^{2+}$ influx and/or $K^+$ efflux across the plasma membrane. PAs assist hydroxyl radicals (·OH) in the activation of nonselective conductance, permeable for cations and small anions (ROSIC), and both ROS and PAs activate the $Ca^{2+}$- and alter the $H^+$-pumping across the plasma membrane. Possible implications for the stress tolerance of ion transport modulation by polyamines and their catabolites are discussed.

**Keywords** $Ca^{2+}$-ATPase • $H^+$-ATPase • Ion channel • Plasma membrane • Polyamine • Reactive oxygen species • Stress • Vacuole

## 19.1 Introduction

In animal cells, polyamines (PAs) act on ion channels mainly as pore "plugs." For instance, voltage-dependent block by cytosolic PAs underlies the inward rectification of the Kir-type $K^+$ channels lacking the intrinsic voltage sensor (Lopatin et al. 1994; Kurata et al. 2010). Additional targets for PAs action in animal cells involve a variety of cation channels in cell and intracellular membranes; in most cases PAs

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were proved to pass through the whole pore of these channels (Drouin and Hermann 1994; Uehara et al. 1996; Williams 1997; Lu and Ding 1999; Huang and Moczydlowski 2001). In addition to a direct pore block, in some cases PAs caused more complex effects via binding to allosteric sites. These include a modulation of the affinity to natural agonists and cofactors or other interference with the channel gating (Williams 1997; Xie et al. 2005; Ahern et al. 2006).

In plants the emerging picture of the PAs action on ion channels appears to be different. Although two types of nonselective cation channels in intracellular (vacuolar) membrane turned to blocked by PAs with a high affinity (Dobrovinskaya et al. 1999a; Pottosin and Muñiz 2002), plasma membrane (PM) cation and $K^+$-selective channels in plants are affected only by relatively high PA concentrations and seemingly indirect. In addition to the effects of PAs themselves, the catabolism of PAs, causing the $H_2O_2$ production, affected the PM ion transport in a reactive oxygen species (ROS)-dependent fashion. In this review, I describe the mechanisms of the PAs block of vacuolar ion channels and provide an overview of the PAs and ROS interplay in the modulation of passive and active cation transport across the PM of plant cells.

## 19.2 Polyamines Exert High-Affinity Block of Vacuolar Cation Channels

FV (fast) and SV (slow) vacuolar channels are ubiquitous in higher plants. SV channels in plants are encoded by TPC1 (two-pore channel 1) (Peiter et al. 2005); the molecular identity of FV channels is still unknown. Both channels conduct small monovalent cations almost indiscriminately (Brüggemann et al. 1999a; Pottosin and Muñiz 2002; Pottosin and Dobrovinskaya 2014). The SV channel also conducts alkali earth cations including $Ca^{2+}$, whereas FV ones are suppressed by cytosolic $Ca^{2+}$ and $Mg^{2+}$ (Tikhonova et al. 1997; Brüggemann et al. 1999b; Pottosin et al. 2001; Pottosin and Muñiz 2002).

Brüggemann et al. (1998) found out that cytosolic PAs inhibited the FV channels activity in a reversible, dose-dependent, and voltage-independent manner, without significant effect on the single channel current. For FV channels from barley mesophyll or sugar beet vacuoles, the relative inhibition potency decreased with a decrease of positive charge: (Spm) > spermidine (Spd) ≫ putrescine (Put), with the apparent $K_d$ about 6 μM, 80 μM, and 4 μM, respectively (Brüggemann et al. 1998; Dobrovinskaya et al. 1999a).

Dobrovinskaya et al. (1999a, b) also analyzed the effect of PAs on the SV channel. PAs, applied from either side of the membrane, did not affect the SV open probability, but suppressed the $K^+$ current across the open channel pore in a strongly voltage-dependent manner. Voltage dependence of the block was asymmetrical: it was three times steeper when the PA was applied from the cytosolic side as compared to the vacuolar application, which implies a longer electrical distance traversed by a PA to its docking position within the pore when it enters from the cytosolic side (Fig. 19.1).

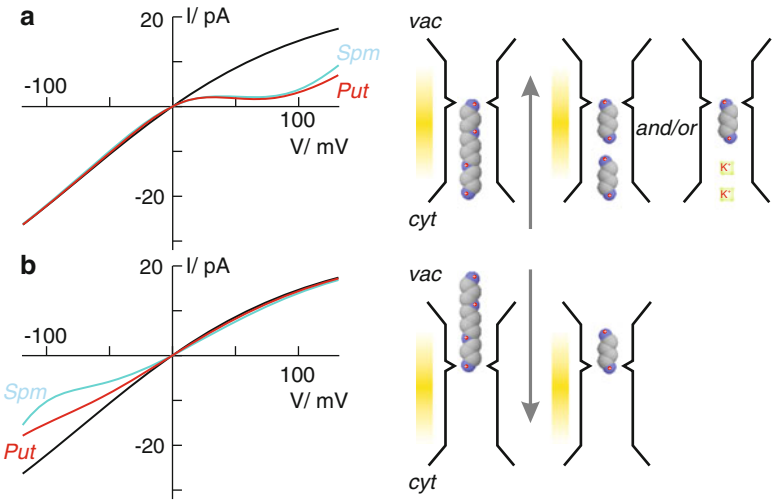


**Fig. 19.1** Mechanism of the SV channel blockage by cytosolic and vacuolar polyamines. Spermine (30 **μ**M) or putrescine (1 mM) were applied at the cytosolic (**a**) or luminal (**b**) tonoplast side, on the background of symmetrical 100 mM KCl. Respective single channel current voltage relationships in control conditions or in the presence of Spm or Put are shown at the *left*, and mechanistic view of the blocked pore state is shown at the *right*. *Arrow* indicates the direction of polyamine (PA) movement across the SV channel pore; *yellow bars* indicate approximate position of the region, where most of the entire voltage drop across the membrane occurred

Blockage by PAs from either side showed relief at large potentials, implying PAs permeation. PAs have a cross-sectional diameter of 0.4–0.5 nm (Weiger et al. 1998) and the SV channel pore is ~0.7 nm at its narrowest cross section (Pottosin and Schönknecht 2007). Theoretical analysis showed that the voltage dependence of the blockage relief was determined by a total number of charges crossing the pore in the full cycle of blockage and relief of block. When added from the vacuolar side, Spm (4+) and Put (2+) displayed the same voltage dependence for blockage, but a rather different one for the block relief (Fig. 19.1b). These data yielded several conclusions: (1) position of charged amines within electrical field in the docking conformation, reached from the vacuolar side, is identical for Put and Spm; (2) two terminal charges of Spm are located outside the transmembrane voltage drop; (3) the total number of positive charges transferred through the entire pore coincided with a blocker charge, +2 and +4 for Put and Spm, respectively. In the case of the permeable block by the cytosolic Spm the number of transported charges coincided with that of Spm. However, the same number of charges (+4) was required to fit the permeable block by cytosolic Spd (+3) or Put (+2). This extra charge clearly reflected the effect of a long pore, which can adopt several ions at a time. Thus, to account for the extra charge in the case of cytosolic Put block one needs to allow the multi-ion occupancy; extra ions may be provided by a second Put or a pair of $K^+$ ions (Fig. 19.1a). The presence of extra charges was revealed also in the case of the pore-permeable block by TMA or Tris, but only when these compounds were added from the cytosolic side;

the blockage from the vacuolar side obeyed the single ion occupancy condition (Dobrovinskaya et al. 1999b). Basing on the voltage dependence of the SV blockage by different amines, the length of the pore constriction, where the most of the electric potential drop occurs, of 2 nm or about the length of Spm was approximated (Pottosin and Schönknecht 2007). At physiological (~0 mV) voltages, apparent $K_d$ values for the SV channel block by cytosolic PAs were 50 μM (Spm) < 500 μM (Spd) < 3 mM (Put) (Dobrovinskaya et al. 1999b).

At extreme $K^+$ deficiency, a relatively constant cytosolic $K^+$ level is maintained at the expense of the vacuolar $K^+$ pool (Walker et al. 1996). This action caused the inversion of the electrochemical gradient for $K^+$ across the tonoplast, from the cytosol-directed to the vacuole-directed one, so that the vacuolar $K^+$ release became active, and vice versa, $K^+$ re-uptake by a vacuole was downhill. Obviously, a futile and energy-consuming $K^+$ cycling between the vacuole and the cytosol needs to be minimized. At $K^+$ deficiency a huge (up to 10 mM) accumulation of Put is observed (Sarjala 1996; Watson and Malmberg 1996). At this condition the activity of FV and SV channels would be efficiently suppressed by the accumulated Put, thus reducing $K^+$ re-uptake into the vacuole (Fig. 19.2). On the other hand, the salt stress is associated

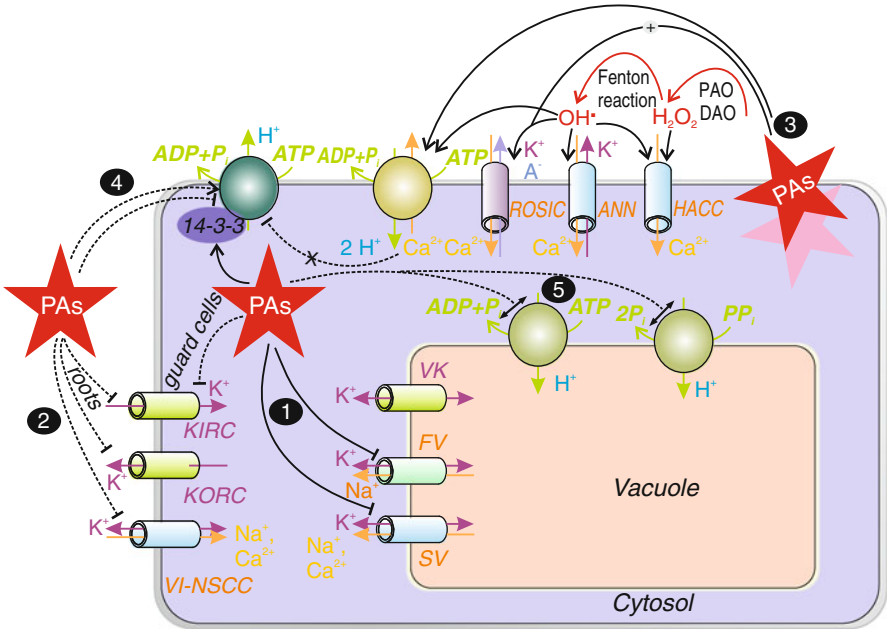


**Fig. 19.2** Summary of polyamine effects on ion transport across the plasma and vacuolar membranes in plants. *1*. PAs directly and with a high affinity inhibited nonselective FV channels, whereas $K^+$-selective VK channels are almost unaffected. *2*. PAs caused an indirect low-affinity inhibition of the PM $K^+$ (inward and outward rectifying, KIRC and KORC) and weakly voltage dependent nonselective cation (VI-NSCC) channels. *3*. ROS-mediated effects of PAs on the PM ion channels; ROS production is initiated by PAs export to the apoplast and oxidation by polyamine oxidases (PAOs) and/or diamine (DAOs) oxidases, yielding $H_2O_2$. ROS activate hyperpolarization-activated $Ca^{2+}$

with the increase of Spm and Spd levels, which in many cases was correlated with the stress resistance (Alcázar et al. 2010). This increase was not as huge as for the Put under the $K^+$ deficiency, but sufficient to cause a significant block of SV channels, and almost a complete suppression of the FV current. On the other hand, $K^+$-selective VK channels are relatively insensitive to PAs at these concentrations (Hamamoto et al. 2008). Thus, the increase of Spm and Spd content, by a preferential suppression of nonselective cation channels of FV and SV types, would increase the overall $K^+/Na^+$ selectivity of the tonoplast conductance. This change will assist the efficient accumulation and prevent the undesired $Na^+$ leak from the vacuole without restriction of the $K^+$ release via $K^+$-selective VK channels (Fig. 19.2). The latter may serve as a shunt conductance for vacuolar $H^+$ pumps and compensate for the salt-induced cytosolic $K^+$ loss, thus improving the plant salt tolerance (Pottosin et al. 2003, 2014; Wang et al. 2013).

## 19.3 Plasma Membrane $K^+$ and Nonselective Cation Channels are Indirectly Affected by Polyamines

Plants do not possess structural homologues of animal Kir channels. Instead, voltage-gated $K^+$ channels in plants, both inward and outward rectifiers, belong to the *Shaker* family (see Sharma et al. 2013 for a recent review), which in animals is represented exclusively by depolarization-activated $K^+$ channels. Nevertheless, inward rectifying $K^+$ channels in the plant plasma membrane appear to be sensitive to PAs, but in a different manner. Inward rectifying $K^+$ current in guard cells is composed of AKT1, AKT2, KAT2, and KAT1 subunits, dominated by the latter current (Sharma et al. 2013). Intracellular PAs with a similar affinity (Spm ~ Spd ≥ Put) inhibited the $K_{in}$ in faba bean guard cells and induced the stomata closure (Liu et al. 2000). As Spd was a dominant PA species in leaves and also the one significantly increased (up to 1 mM) by drought, it was subjected to a more detailed analysis. The effect of Spd on the $K_{in}$ was voltage independent, but dose dependent, with apparent $K_d$ about 0.6 mM, very similar to a concentration needed to produce a half-effect on the stomata aperture. Similar effect of the Spd was also demonstrated for the homomeric KAT1 current, overexpressed in tobacco mesophyll. Therefore, it was

**Fig. 19.2** (continued) influx channels (HACC), annexin (ANN), and weakly voltage-dependent nonselective conductance (ROSIC). ROSIC activation is potentiated by PAs. Hydroxyl radicals and PAs activate the PM $Ca^{2+}$ pump, which may relieve the inhibition of the $H^+$-pumping PM ATPase by cytosolic $Ca^{2+}$. *4.* Multiple effects of PAs on the PM $H^+$-ATPase are likely indirect. Both translational and posttranslational regulations are involved. The only specific mechanism described so far is the activation of the $H^+$-ATPase activity by promotion of the 14-3-3 protein binding to the unphosphorylated enzyme. *5.* Only effects of long-lasting incubations with PAs were reported for the vacuolar $H^+$ pumps and their mechanisms are elusive, although there were attempts to link them with the impact of PAs on the membrane structure

concluded that the drought-induced increase in PAs (here, Spd) favored stomata closure, thus reducing transpiration and water loss by a plant (Liu et al. 2000). Effect of the Spd on the $K_{in}$ in guard cells was only observed when this PA was introduced into the cell via patch pipette; no effect was observed upon the extracellular application of the Spd. However, when the Spd was applied to an excised inside-out membrane patch, neither single $K^+$ channel currents nor open probability was affected. This observation implies that the Spd acted on the $K^+$ channel from the intracellular side, but its effect was indirect and mediated by some unidentified components, which were lost upon the patch excision. In roots, inward rectifying $K^+$ channels are represented by AKT1 homologues. In epidermal, cortical, and xylem parenchyma cells of barley roots, *extracellular* but not *intracellular* application of 1 mM Spd caused approximately a twofold decrease of the inward $K^+$ current (Zhao et al. 2007). Spm caused a slightly higher and Put a slightly lower effect on the $K^+$ inward current. We (Zepeda-Jazo and Pottosin, unpublished data) confirmed the data by Zhao et al. (2007), testing the effects of 1 mM Put or Spm on $K^+$ currents in barley root epidermis. In addition to the inhibition of the inward rectifying $K^+$ channels, PAs with approximately the same potency (as an average, half-inhibition by 1 mM PA) caused also the inhibition of the outward rectifying $K^+$ current, which in root epidermis and cortex is mediated by guard cell-type outwardly rectifying $K^+$ (GORK) channels, although the effects of PAs on both outward- and inward rectifying channels in roots displayed a high degree of variability with respect to the extent of the inhibition and to its reversibility. Therefore, it seems highly improbable that PAs plugged the $K^+$ channel pore. More likely, PAs effects on inward- and outward rectifying $K^+$ channels were indirect and mediated by some cellular components.

In addition to truly selective $K^+$ channels, in plant plasma membrane there is a variety of nonselective cation channels (NSCCs), conducting $K^+$ and $Na^+$ almost indiscriminately (Demidchik and Maathuis 2007). A prototypic weakly voltage-sensitive instantaneous channel (VI-NSCC) was described first in *Arabidopsis* roots and, basing on the comparison of its pharmacology with that of the low-affinity $Na^+$ uptake, it was considered as an important mediator of the toxic $Na^+$ influx (Demidchik and Tester 2002). Similar current in barley roots was inhibited by extracellular PAs (1 mM), from 30 % to 75 %, with the effect of Spm being the strongest among PAs. Inhibition of the VI-NSCC by PAs correlated with a decrease of the accumulation of $Na^+$ in the shoot and alleviated the salt stress-induced injury (Zhao et al. 2007). In pea leaves, the VI-NSCC inhibition by external PAs, Put, or Spm with a similar potency, correlated with decreases in the salt-induced membrane depolarization and $K^+$ efflux (Shabala et al. 2007). The effect of PAs on the VI-NSCC, albeit reversible, developed slowly (several minutes). There are two possible explanations: (i) PAs acted from the cytosolic side, and their uptake into the cell required a substantial time, or (ii) the PAs effect on the VI-NSCC was indirect. As Zhao et al. (2007) did not find any significant effect of PAs from the cytosolic side on the VI-NSCC, the latter possibility seems more plausible.

Based on the effects of PAs on the outward rectifying $K^+$ channels, the main route for the NaCl-induced $K^+$ efflux (Shabala et al. 2006; Chen et al. 2007), and on

the VI-NSCC, a simple hypothesis, explaining beneficial effects of PAs on plant salt tolerance, was proposed. According to that, PAs reduced $Na^+$ influx through the NSCC, thus reducing the $Na^+$-induced depolarization and driving force for the $K^+$ loss, in addition to the reduction of the $K^+$ efflux through partly suppressed GORK and NSCC (Zepeda-Jazo et al. 2008). This hypothesis was addressed in further studies. As salt tolerance in a variety of plant species strongly correlated with the $K^+$ retention (Chen et al. 2007; Cuin et al. 2008; Sun et al. 2009), Pandolfi et al. (2010) evaluated the magnitude of the NaCl-induced $K^+$ efflux in roots of maize and *Arabidopsis*, in the presence or absence of PAs in the external medium. Results of this study did not agree with the predictions of the simple model. Depending on plant and PA species, growth conditions, and root zone, NaCl-induced $K^+$ efflux could be suppressed, unaffected or, notably, enhanced in the presence of PAs. The latter is especially curious, because instead of the inhibition of the PM channels it implies their stimulation by PAs. Therefore, the original hypothesis needed to be rethought and additional effects of PAs on ion channels, not restricted to their inhibitory effects, had to be considered.

## 19.4 Polyamines are Crosstalking with Reactive Oxygen Species in the Regulation of Ion Transport Across the Plasma Membrane

Recent studies on plants revealed an interesting signaling mechanism, including the exodus of PAs to the space between the PM and cell wall, their oxidation by apoplastic amine oxidases, generating $H_2O_2$ and other reactive oxygen species (ROS), which induced a variety of ion conductances, including those nonselective and $Ca^{2+}$ permeable, in the PM (Fig. 19.2) (see also Pottosin et al. 2014 for a recent review). This mechanism is implemented in plant–pathogen interactions (Yoda et al. 2006; Marina et al. 2008), responses to wounding (Tisi et al. 2008) and salt (Rodríguez et al. 2009) stresses, stomatal movements (An et al. 2008), and polarized growth (Wu et al. 2010). When it comes to ROS-activated currents, those activated by hydroxyl radicals (·OH) in addition to the $Ca^{2+}$ influx mediated also the efflux of $K^+$ (Demidchik et al. 2003, 2010; Zepeda-Jazo et al. 2011; Laohavisit et al. 2012). ·OH generation in the extracellular space (Fig. 19.2) represents a crucial component for organ growth and early response to stresses (Foreman et al. 2003; Demidchik et al. 2010; Pottosin et al. 2014). In pea and barley roots, ·OH induced a weakly voltage-dependent conductance, permeable for different cations and small anions, termed ROSIC (Zepeda-Jazo et al. 2011; Velarde-Buendía et al. 2012). External PAs (1 mM) by themselves hardly induced ROSIC, but can potentiate it, so that the joint application of a PA and ·OH treatment to intact roots or isolated root protoplasts could increase ROSIC severalfold (Fig. 19.2). Effects of different PAs were comparable. Isolated protoplasts lack extracellular amine oxidases, excluding the mechanism mediated by PA oxidation. Most likely, PAs act as cofactors for the ROSIC or

positively interact with its cofactors, as in animal cells PAs sensitize the TRPV1 channels to capsaicin (Ahern et al. 2006) or stabilize the binding of the $PIP_2$ to the Kir2.1 channel (Xie et al. 2005). In plant roots, the synergistic action of PAs and ·OH is restricted to the mature zone and was more pronounced in a salt-sensitive as compared to a salt-tolerant variety, emphasizing the role of the $K^+$ retention as a key determinant for salt tolerance (Pottosin et al. 2012; Velarde-Buendía et al. 2012).

Externally applied polyamines rapidly induced biosynthesis of NO in plants, mediated by $H_2O_2$ or otherwise involving PAs and the activity of polyamine and/or diamine oxidases (Tun et al. 2006; Wimalasekera et al. 2011). NO causes the inhibition of GORK by a direct nitrosylation (Sokolovski and Blatt 2004) and a two- to threefold stimulation of the PM $H^+$-ATPase activity (Zandonadi et al. 2010). NO also caused the intracellular $Ca^{2+}$ release, thus inhibiting the inward rectifying $K^+$ and activating the slow anion current in guard cells, promoting stomata closure (García-Mata et al. 2003).

Unexpectedly, PAs (1 mM) or ·OH caused a rapid (within 1 min) stimulation of $Ca^{2+}$ pumping across the PM in intact roots, sensitive to the eosin yellow-specific inhibitor of the $Ca^{2+}$-pumping ATPases (Bose et al. 2011; Zepeda-Jazo et al. 2011; Velarde-Buendía et al. 2012). In animal cells, in contrast, PAs tend to inhibit $Ca^{2+}$-ATPase by affecting the $Ca^{2+}$-binding and transport step, and decreasing the $Ca^{2+}$-ATPase phosphorylation level by competition with $Mg^{2+}$ (Hughes et al. 1995 and references therein). Put or Spm produced the same $Ca^{2+}$ efflux in pea roots (Bose et al. 2011; Velarde-Buendía et al. 2012), but caused contrasting effects on the $H^+$ fluxes: an efflux or an influx, respectively (Velarde-Buendía 2013). PM $Ca^{2+}$-ATPase appears to import two $H^+$ per each exported $Ca^{2+}$ (Beffagna et al. 2000), in agreement with observed magnitudes of Spm-induced $Ca^{2+}$ and $H^+$ fluxes. The vanadate-sensitive $H^+$ efflux, induced by Put, however, implied the activation of the PM $H^+$ pump. This activation could be indirect, through a decrease of the cytosolic $Ca^{2+}$, which inhibits the $H^+$ pump activity (Kinoshita et al. 1995; Brault et al. 2004). The observed effect of the $Ca^{2+}$ pump inhibitor on the Put-induced $H^+$ fluxes corroborated the view that the $H^+$ pump activation is coupled to the activation of the PM $Ca^{2+}$ pump (Velarde-Buendía 2013). In maize roots, the Spm but not shorter PAs promoted 14-3-3 protein binding to the unphosphorylated PM $H^+$-ATPase, thus relieving the mechanism of autoinhibition and promoting the $H^+$-ATPase activity (Garufi et al. 2007). On the other hand, salt-induced $H^+$ pumping by the vanadate-sensitive PM ATPase in maize roots was suppressed by PAs, especially, by Spm (Pandolfi et al. 2010). Increase of the $H^+$-ATPase activity and decrease of the $H^+$ pumping are not necessarily contradictory, as the $H^+$-ATPase is partly uncoupled, transporting less than one $H^+$ per ATP hydrolyzed, and such uncoupling can be regulated by cation binding (Gaxiola et al. 2007). All PAs indiscriminately stimulated the $H^+$-ATPase activity in rice roots (Reggiani et al. 1992). As anoxia resulted in a massive increase of Put (Reggiani et al. 1993) and caused the cytosolic acidification (Felle 2005) it is tempting to propose that the Put-induced $H^+$-ATPase activation may tend to counteract the acidification trend.

In addition to immediate effects of PAs on the P-type $Ca^{2+}$- and $H^+$-ATPases, there are others, developed in the course of one to several days. Treatment with Put (0.05 mM) increased the levels of higher PAs and of the $Ca^{2+}$-ATPase activity in carrot culture cells (Sudha and Ravishankar 2003). Salt stress caused decrease of the activity of the PM and vacuolar $H^+$ pumps in rice and barley roots, respectively, which can be substantially reversed in the presence of PAs (Sun et al. 2002; Roy et al. 2005). In contrast, salt-treated virgin pine calli and plantlets display an increased activity of the vacuolar $H^+$-ATPase, and PAs again tended to reduce these changes (Tang and Newton 2005). In cucumber roots intracellular PAs had no direct effect on activity of the PM $H^+$-ATPase, but 1-day treatment of plants with 0.05 mM PAs caused a threefold decrease in the transcript level of one of the PM $H^+$-ATPase isoforms and substantially lower $H^+$ pumping rates (Janicka-Russak et al. 2010).

## 19.5 Conclusions and Future Perspective

Vacuolar SV and FV channels are already partly blocked by cytosolic PAs at resting conditions, so variations of PAs levels during stresses would further modulate the channel-mediated currents. As the SV channel identity is known, and the pore sequence may be deduced, the search of amino acid residues, forming the PA-binding site, could be a proximate task. With respect to the channel physiological role, which is still disputed (Pérez et al. 2008; Hedrich and Marten 2011), it would be interesting to see phenotypes of the mutants with increased or decreased affinity for the PAs binding. To date, FV channels are the most sensitive to cytosolic Spm and Spd among plant ion channels. As FV channels may dominate the tonoplast conductance at resting cytosolic $Ca^{2+}$ (Pottosin and Muñiz 2002), it justifies further attempts to unravel the identity of these channels; this will gain a possibility to manipulate genetically with the activity/expression of both major cation currents of the tonoplast. For instance, it has been shown recently that the downregulation of both SV and FV channels underlies the efficient vacuolar $Na^+$ sequestration in leaves of the salt-grown quinoa (Bonales-Alatorre et al. 2013). When it comes to the effects of PAs on the PM $K^+$ and nonselective cation channels, their study seems less promising for a short perspective, because the effects of PAs on these channels are not mediated by the pore plugging and are likely indirect. However, effects of PA catabolites on PM ion conductance, in particular, ROS-activated currents, are worth of exploring. Induction of $Ca^{2+}$ influx currents by ROS and $Ca^{2+}$ pumping by ·OH or PAs opens an interesting perspective for $Ca^{2+}$ signaling. Also, the nature and role of the ROSIC, the dual cation and anion conductance in the PM, induced by ·OH and potentiated by PAs, are of considerable interest.

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# Chapter 20
# Regulation of *N*-Methyl-D-Aspartate Receptors by Spermine and Ifenprodil

**Keiko Kashiwagi, Keith Williams, and Kazuei Igarashi**

**Abstract** *N*-Methyl-D-aspartate (NMDA) receptors are a subtype of glutamate receptor. They are tetramers containing combinations of GluN1, GluN2, and GluN3 subunits. Activation of NMDA receptors is associated with the induction of synaptic plasticity including long-term potentiation and depression, processes that may underlie learning and memory. Excessive activation of NMDA receptors is also involved in neurodegeneration. Spermine has multiple effects on NMDA receptors, including stimulation and a weak voltage-dependent channel block; these effects involve binding of spermine to at least two distinct sites. Ifenprodil is an atypical NMDA receptor antagonist that selectively inhibits receptors containing the GluN2B subunit. Modeling the binding sites for spermine and ifenprodil on the regulatory (R) domains of NMDA receptor GluN1 and GluN2B subunits was carried out after measuring spermine stimulation and ifenprodil inhibition at receptors containing GluN1 and GluN2B R domain mutants. The modeling suggests that a space between the two R1 lobes of GluN1R and GluN2BR is promoted through binding of spermine, and that R1 lobes of GluN1R and GluN2BR come closer after binding of ifenprodil, an effect opposite to that seen after binding of spermine. The spermine-binding site involved in the channel block was also localized at the pore-forming and vestibule regions of NMDA receptors.

**Keywords** Channel blockers • Glutamate receptors • Ifenprodil • Memantine • NMDA receptors • R (Regulatory) domain • Spermine • Tri-benzylspermidine

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## 20.1 Introduction

*N*-Methyl-D-aspartate (NMDA) receptors are a subtype of ligand-gated glutamate receptors, which also include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Traynelis et al. 2010). Although they share common structural features, most NMDA receptors have characteristics different from AMPA and kainate receptors: (1) not only glutamate but also glycine is necessary for activation of NMDA receptors; (2) NMDA receptors show high permeability to $Ca^{2+}$; and (3) NMDA receptor activity is blocked by $Mg^{2+}$ at the resting membrane potential and the block is relieved by depolarization. Most NMDA receptors in the adult central nervous system are tetramers that contain combinations of two GluN1 and GluN2 subunits, with GluN2A and GluN2B predominating in forebrain areas such as the cerebral cortex (Hollmann and Heinemann 1994; Traynelis et al. 2010; Watanabe et al. 1992). Each subunit contains a distal amino-terminal domain (Perin-Dureau et al. 2002), also referred to as the regulatory (R) domain (Masuko et al. 1999), agonist-binding (S) domain, channel-forming domain, and intracellular domain (Fig. 20.1). There are three membrane-spanning regions (M1, M3, M4), and a segment (M2) that forms a re-entrant loop and contributes to the narrowest constriction of the channel (Fig. 20.1). The M2 loop region in GluN1 and GluN2 subunits is a critical determinant of divalent cation permeability and $Mg^{2+}$ block. In particular, asparagine residues in this region form part of the $Mg^{2+}$-binding site and contribute to the selectivity filter of the channel (Traynelis et al. 2010).

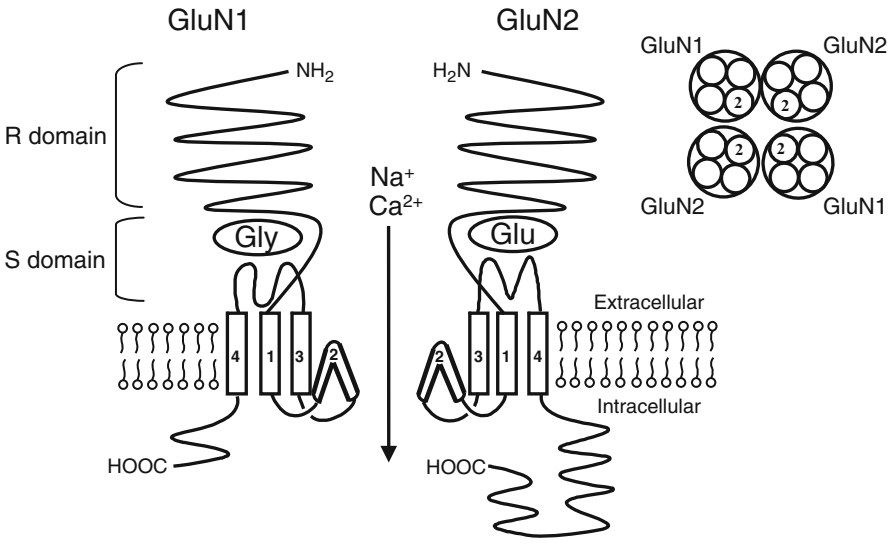


**Fig. 20.1** Schematic showing the structure of NMDA receptor subunits. NMDA receptors are tetramers, with the M2 region of each subunit contributing to the channel pore. Portions of the M1, M3, and M4 domains also form part of the ion channel pore and vestibule

**a**

| | NMDA receptor | | | |
|---|---|---|---|---|
| | 1/2A | 1/2B | 1/2C | 1/2D |
| Effect of spermine | | | | |
| 1. Glycine independent stimulation | — | + | — | — |
| 2. Glycine dependent stimulation | + | + | — | — |
| 3. Voltage dependent inhibition | + | + | — | — |
| Ifenprodil inhibition | weak | strong | weak | weak |

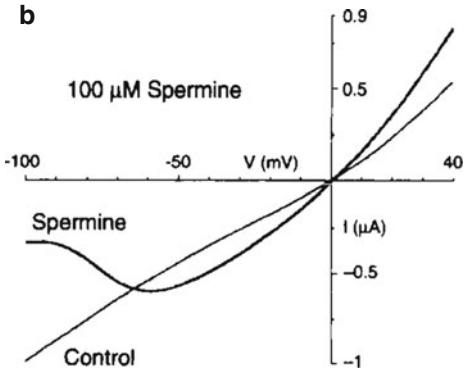


**Fig. 20.2** Effects of spermine and ifenprodil on NMDA receptors. **a** Spermine has three macroscopic effects that are differentially influenced by GluN2 subunits. **b** Schematized current–voltage (I-V) plots for NMDA (GluN1/GluN2B) receptors. Spermine (100 μM) potentiates NMDA receptors at depolarized membrane potentials and inhibits them at hyperpolarized membrane potentials

Polyamines, in particular spermine, have multiple effects on NMDA receptors, including stimulation and a weak voltage-dependent inhibition resulting from open-channel block (Benveniste and Mayer 1993; Igarashi and Williams 1995; Williams 1997; Williams et al. 1995) (Fig. 20.2). Thus, the spermine-binding sites, which are involved in stimulation and voltage-dependent inhibition by spermine, were characterized by measuring effects of spermine on NMDA receptors containing various GluN1 and GluN2B mutants. The effects of ifenprodil, a potent inhibitor of NMDA receptors containing the GluN2B subunit, were also studied (Masuko et al. 1999; Perin-Dureau et al. 2002). Excessive or abnormally prolonged activation of NMDA receptors can lead to neurodegeneration (Choi 1988; Olney 1990). Thus, we also searched for polyamine derivatives that exhibit a strong channel block of NMDA receptors and may be lead compounds for agents to reduce or prevent NMDA receptor-induced neurotoxicity.

## 20.2 Spermine- and Ifenprodil-Binding Sites on R Domain

We have identified a spermidine-binding site on PotD protein, a spermidine-binding protein in the spermidine preferential transport system in *Escherichia coli*, by X-ray analysis of the crystal structure and measurement of activities of various mutated PotD proteins (Kashiwagi et al. 1996; Sugiyama et al. 1996). Thus, for NMDA receptors, the strategy we initially adopted was centered around a region of GluN1 that shares amino acid sequence homology with PotD. Mutant GluN1 subunits, expressed together with GluN2B in heteromeric GluN1/GluN2B receptors, were studied by voltage-clamp recording in *Xenopus* oocytes. It was found that E342 residue in GluN1, which is located in the R domain, was found to be critical for glycine-independent spermine stimulation (Williams et al. 1995). We also found that the E181 residue in GluN1 is involved in spermine stimulation (Masuko et al. 1999). Aromatic amino acid residues such as F, Y, and W are also involved in spermidine binding to PotD (Kashiwagi et al. 1996; Sugiyama et al. 1996). Thus, acidic and aromatic amino acid residues in GluN1 were mutated to neutral residues (e.g., E to Q, D to N, and F, Y, or W to L), and the activity of mutated NMDA receptors was measured (Tomitori et al. 2012). As shown in Fig. 20.3, several amino acid residues were involved in spermine stimulation and ifenprodil inhibition. Modeling the binding sites for spermine and ifenprodil on the R domains of GluN1 and GluN2B was carried out. A binding site for spermine was formed by residues near the cleft between the R1 (N-terminal side) and R2 (C-terminal side) lobes of the GluN1 R domain together with residues on the surface of the R2 lobe of the GluN2B R domain. The ifenprodil binding site included residues on the surface of the R1 lobe of GluN1R together with residues near the cleft between the R1 and R2 lobes of GluN2BR (Fig. 20.4). It was confirmed using a Western blotting analysis that GluN1R and GluN2BR formed a heterodimer (Han et al. 2008). Thus, models of spermine and ifenprodil binding to the heterodimer were constructed based on the published crystal structure of the GluN1 and GluN2B R domains (Karakas et al. 2011). The modeling suggests that an open space between the two R1 lobes of GluN1R and GluN2BR is promoted through binding of spermine and that the R1 lobes of GluN1R and GluN2BR come closer after binding of ifenprodil, an effect opposite to that seen with binding of spermine (Fig. 20.5). The sensitivity of the GluN1R-GluN2BR heterodimer to trypsin was increased in the presence of spermine to a greater extent than in the presence of ifenprodil. This finding is consistent with the idea that the spermine-GluN1R/GluN2BR complex is more relaxed than the ifenprodil-GluN1R/GluN2BR complex. The $K_m$ values for the binding of spermine to GluN1R and GluN2BR were 19 and 33 μM, respectively, and those for the binding of ifenprodil to GluN1R and GluN2BR were 0.18 and 0.21 μM, respectively (Han et al. 2008).

It is necessary to accumulate spermine in synaptic vesicles if spermine functions as a neuromodulator in the central nervous system. Spermine content in whole cerebrum and synaptic vesicles was 3.1 and 8.3 nmol/mg protein, respectively, and it was shown that spermine is released from rat hippocampal slices by depolarization using a high concentration of KCl (Masuko et al. 2003).

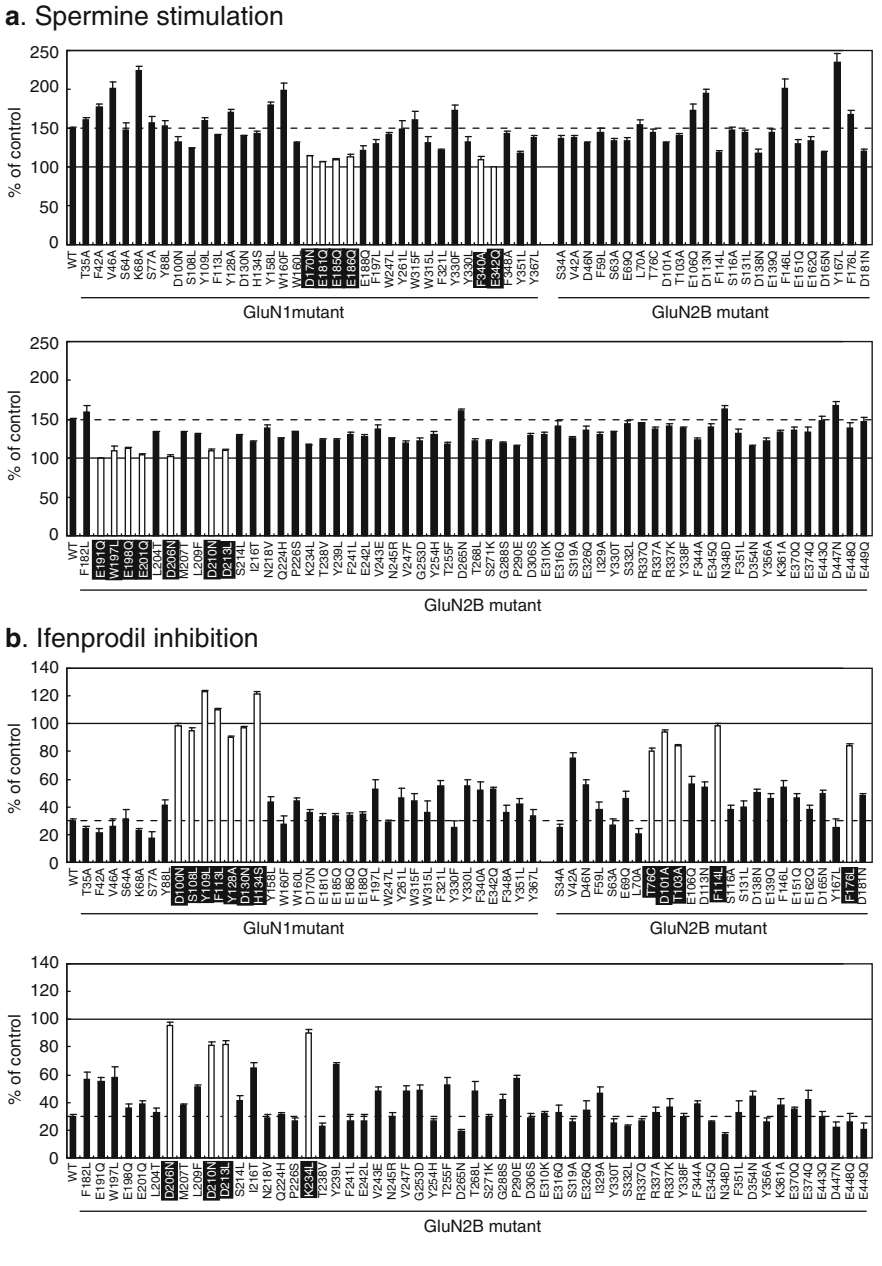


**Fig. 20.3** Effects of spermine (**a**) and ifenprodil (**b**) at GluN1/GluN2B receptors containing GluN1 and GluN2B mutants. The effects of spermine (100 μM) and ifenprodil (1 μM) were determined in oocytes expressing GluN1/GluN2B receptors with wild-type (WT) and mutant GluN1 or GluN2B subunits, voltage clamped at −20 mV and activated by 10 μM glycine and 10 μM glutamate. Mutants shown with *white letters* in a *black box* were involved in spermine stimulation and ifenprodil inhibition

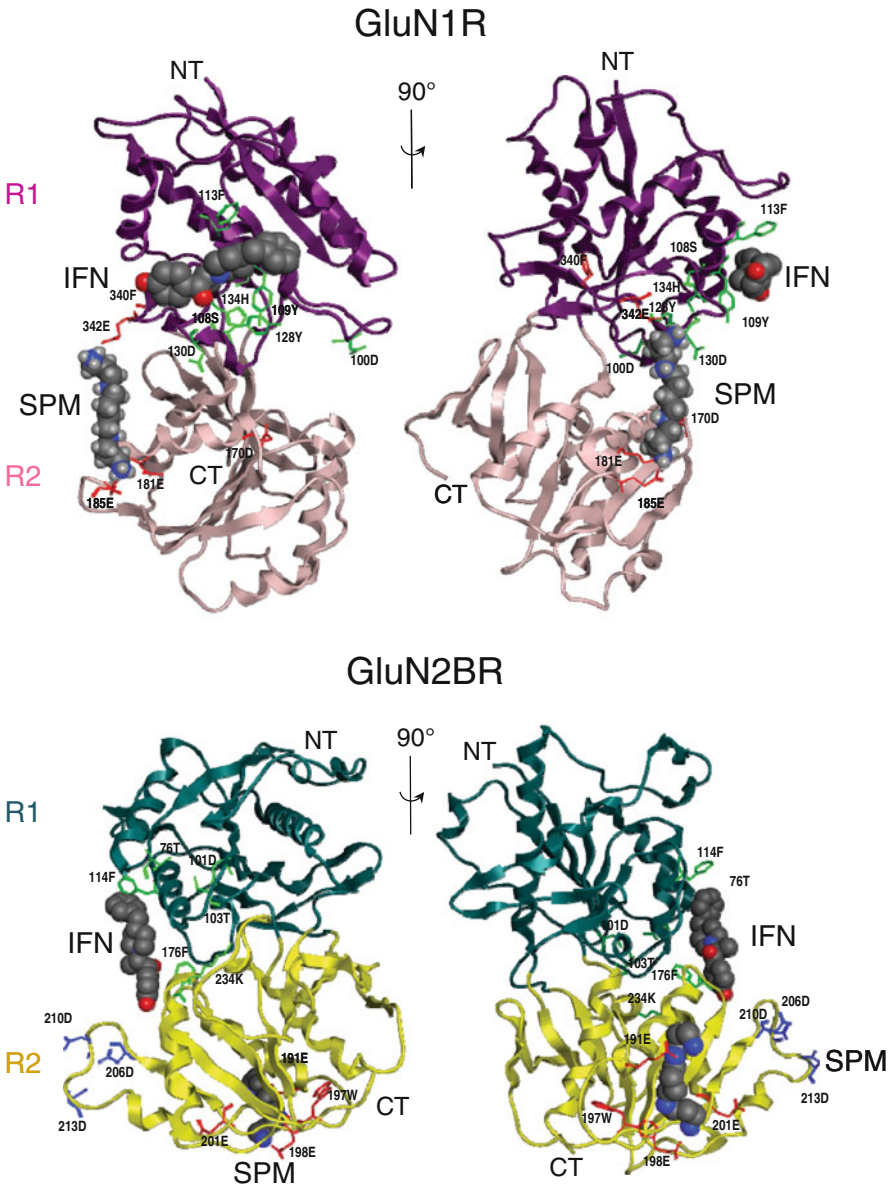


**Fig. 20.4** Modeling of spermine and ifenprodil binding to GluN1R and GluN2BR monomers. Spermine and ifenprodil binding to GluN1R and GluN2BR monomers is shown from two different angles, rotated by 90°. *SPM* spermine, *IFN* ifenprodil, *NT* N terminus, *CT* C-terminus, *R1* and *R2* R1 and R2 lobes, respectively

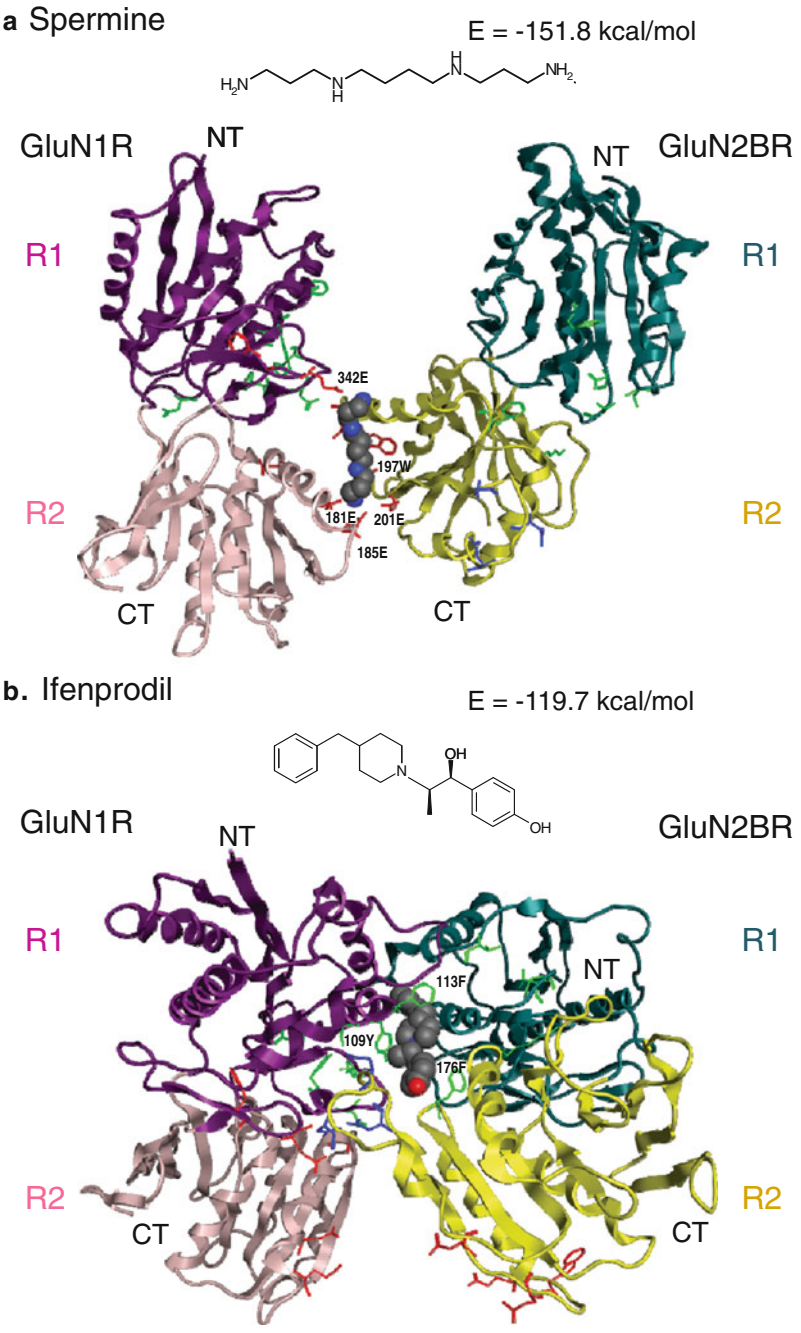


**Fig. 20.5** Modeling of spermine- (**a**) and ifenprodil- (**b**) binding sites on GluN1R and GluN2BR heterodimers shows the approximate orientation of GluN1R and GluN2BR and the interaction energy of these complexes

Taken together, the results suggest that spermine may function as a modulator of NMDA receptors in vivo, potentiating receptor activity at depolarized potentials, and that the binding site for spermine on NMDA receptors is distinct from the binding site for ifenprodil.

## 20.3 Channel Region Influencing Spermine Stimulation and Inhibition

Spermine also functions as a voltage-dependent channel blocker (Fig. 20.2b). To investigate the site and mechanism of action of the voltage-dependent block by spermine, effects of spermine on NMDA receptors were compared at −20 mV and at −100 mV using GluN1 and GluN2B receptors with point mutations in the transmembrane and pore-forming regions. Block by spermine was predominantly affected by mutations in the M3 segment of GluN1 and especially in the M1 and M3 segments of GluN2B together with the M2 loop region of both subunits (Fig. 20.6) (Jin et al. 2008). These regions in M1 and M3 are in the outer vestibule of the channel pore and may contribute to a spermine-binding site. Mutations in different regions, predominantly the M3 segment and M2 loop of GluN1 and the M3 segment of GluN2B, influenced spermine stimulation (Fig. 20.6), a surprising finding because spermine stimulation is thought to involve a spermine-binding site in the distal, extracellular regulatory (R) domain. Some of the residues involved in spermine stimulation in the M1 and M3 segments of GluN1 (P557G, T648A, A649C, A653T, V656A, and L657A) also generate a constitutively open channel (Kashiwagi et al. 2002). The results suggest that the M3 segment of GluN1 is strongly involved in the channel opening together with the R domain. The results are consistent with the proposal that the relative positions of the M1 and M3 transmembrane segments and M2 loops are staggered or asymmetrical in GluN1 and GluN2B subunits (Fig. 20.6) (Beck et al. 1999; Sobolevsky et al. 2007), and with the idea that stimulation and block by spermine involve separate binding sites and distinct mechanisms.

Overactivation of NMDA receptors leads to neuronal cell death. Thus, NMDA receptors are potential targets for neuroprotective agents and anticonvulsants (Choi 1988; Rogawski 1992). We studied polyamine-derived channel blockers of NMDA receptors as potential lead compounds for clinically useful NMDA receptor antagonists. The effects of benzylpolyamines were studied at recombinant NMDA receptors expressed in *Xenopus* oocytes (Igarashi et al. 1997). A number of mono-, di-, and tri-benzyl polyamines, having benzyl substitution on the terminal or central amino groups, inhibited responses of GluN1/GluN2A receptors in oocytes voltage-clamped at −70 mV. NMDA receptors consisting of GluN1/GluN2A were used in these experiments because they do not exhibit stimulation by polyamines. Among these compounds, the most potent compound was $N^1$,$N^4$,$N^8$-tri-benzylspermidine (TB34), which had an $IC_{50}$ value of 0.2 μM. Block by TB34 was strongly voltage

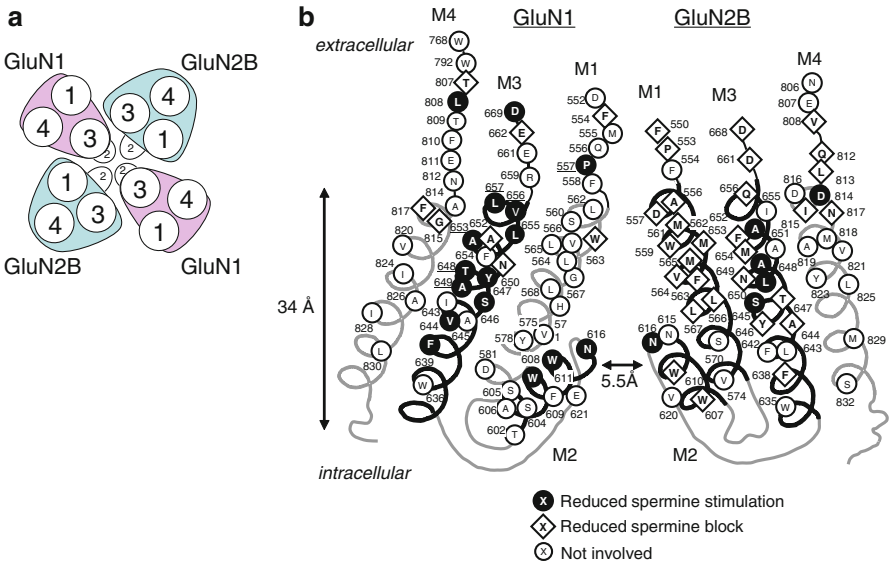


**Fig. 20.6** Models illustrate possible subunit arrangements (**a**) and residues that affect spermine stimulation and block in the channel pore and vestibule of GluN1/GluN2B receptors (**b**). **a** The contribution of each helix of GluN1 and GluN2B to the pore and outer vestibule of the channel is presented. The arrangement is asymmetrical, with both the M1 and M3 segments of GluN2B and, predominantly, the M3 segment of GluN1 contributing to the outer vestibule/entrance to the pore. **b** The M1-M2-M3-M4 region is depicted with a helix-loop-helix (M1-M2-M3) core region similar to the structure reported for the KcsA potassium channel from *Streptomyces lividans* (Doyle et al. 1998). *Numbers* next to each residue indicate the amino acid number from the initiator methionine in each subunit. Numbers *underlined* indicate positions at which mutations generate constitutively open channels (Kashiwagi et al. 2002)

dependent. At a concentration of 10 μM, TB34 had no effect on AMPA receptors, indicating that TB34 is a preferential NMDA receptor antagonist.

The effects of mutations in and around the membrane-spanning and pore-forming regions of NMDA receptors were studied using three channel blockers: TB34, memantine, and MK801. Mutations at the critical aspargine residues in the M2 loop of GluN1 (N616) and GluN2B (N615 and N616) and at a tryptophan residue in the M2 loop of GluN2B (W607) reduced block by three blockers. Furthermore, mutations at A645 and Y647 in the M3 segment of GluN1 also reduced block by three blockers. Mutations at residues in the pre-M1, M1, M3, post-M3, and post-M4 regions had differential effects on these three blockers. Many mutations in these regions reduce block by TB34 and MK801 but had no effect on block by memantine. The benzyl rings in TB34 and MK-801 may make hydrophobic interactions with aromatic and hydrophobic amino acid residues in the channel pore. We also found that anthraquinone- and anthracene-tetraamines are strong blockers of NMDA channels (Jin et al. 2007; Kashiwagi et al. 2007). Memantine is now used as a neuroprotective agent. Some polyamine derivatives may also be lead compounds for neuroprotective agents.

## 20.4 Future Perspectives

Spermine has multiple effects on NMDA receptors, including stimulation and a weak voltage-dependent channel block that involves the binding of spermine to least two sites (Williams 1997). We succeeded in identifying spermine- and ifenprodil-binding sites. Ifenprodil is a specific inhibitor of NMDA receptors containing the GluN2B subunit. Our results indicate that the relative positions of GluN1R and GluN2BR are altered through binding of spermine and ifenprodil: the R1 lobes of GluN1R and GluN2BR are separated in the presence of spermine, but are brought together in the presence of ifenprodil. The difference may lead to the stimulation or inhibition of NMDA receptor activity.

The binding site for spermine that is involved in block of NMDA channels was also characterized in the pore-forming and vestibule regions of NMDA receptors. Block by spermine was predominantly affected by mutations in the M3 segment of GluN1, and especially in the M1 and M3 segments of GluN2B. Our results were consistent with the proposal that the relative position of the M1 and M3 transmembrane segments and M2 loops are staggered or asymmetrical in GluN1 and GluN2B subunits. We also identified several polyamine derivatives such as TB34 that act as strong and selective NMDA channel blockers.

We hope that the following points will be elucidated in the near future: (1) role of the M3 segment of GluN1 on the channel opening of NMDA receptors; (2) elucidation of the effect of spermine on physiological process such as long-term potentiation through activation of NMDA receptors; and (3) development of polyamine derivatives as clinically useful blockers of NMDA receptors.

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# Part IX
# Polyamines and Longevity

# Chapter 21
# Polyamines and Longevity in Mammals

**Mitsuharu Matsumoto**

**Abstract** Polyamines are universally present in all cells of every species. Polyamines have several biological functions, such as in the synthesis and stabilization of DNA, RNA, and proteins; in cell proliferation; and in the maturation and maintenance of intestinal mucosal barriers. They also show antiinflammatory and antimutagenic activities and protect against diverse stresses and autophagy. In mammals, body polyamine levels decrease during the aging process. Polyamines have therefore been found to be useful for the inhibition of chronic inflammation, which is one of the main symptoms of geriatric diseases. In fact, oral polyamine supplementation increases lifespan in various model organisms such as yeast, fly, and worm. Although research on the promotion of longevity using polyamines has just begun in mammals, several effective results have already been obtained. Interestingly, the longevity observed in mice given exogenous polyamines is not the same mechanism of longevity obtained by calorie restriction, which has been shown to increase longevity in a variety of species. In addition, because exogenous polyamines derived from meals are absorbed before they reach the lower parts of the intestine, the greatest amounts of the polyamines in the lower parts of the intestine are synthesized by intestinal microbiota. In this chapter, we provide an overview of the effects of exogenous polyamines, which are supplied by food or by intestinal microbiota altered using probiotics, on longevity.

**Keywords** Inflammation • Intestinal microbiota • Longevity • Polyamine • Probiotics

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## 21.1 Introduction

Many mechanisms have been shown to contribute to the process of senescence of mammals, such as telomere shortening in replicative cells, cumulative DNA damage leading to genomic instability, and oxidative damage to critical molecules by reactive oxygen species (ROS) (Tosato et al. 2007). These mechanisms also comprise chronic low-grade inflammation, a major risk factor for aging and age-related diseases, such as Alzheimer's disease and type II diabetes (Chung et al. 2009; Brüünsgaard and Pedersen 2003). Furthermore, the prevention of chronic low-grade inflammation appears to be one of the most effective approaches for increasing longevity (Franceschi et al. 2007). Polyamines have many functions to protect these causes of senescence. This section introduces the relationship between polyamines and longevity (anti-aging) in mammals.

## 21.2 Polyamines Preserve Healthy Cellular Life

The role of polyamines is to maintain cellular health. Many studies show that polyamines are essential for cell proliferation (Fig. 21.1). One of the main functions of polyamines is to interact with negatively charged molecules, such as DNA, RNA, proteins, and phospholipids (Bachrach 2005), and polyamines are involved in the synthesis and stabilization of DNA, RNA, and protein molecules, as well as in cell proliferation and differentiation and in the regulation of enzymatic activity (Tabor and Tabor 1984; Igarashi and Kashiwagi 2010). Polyamines function as scavenger against diverse stress, such as reactive oxygen species (ROS), heat, ultraviolet, and psychiatric stress (Rhee et al. 2007). Polyamines are also potentially antimutagenic

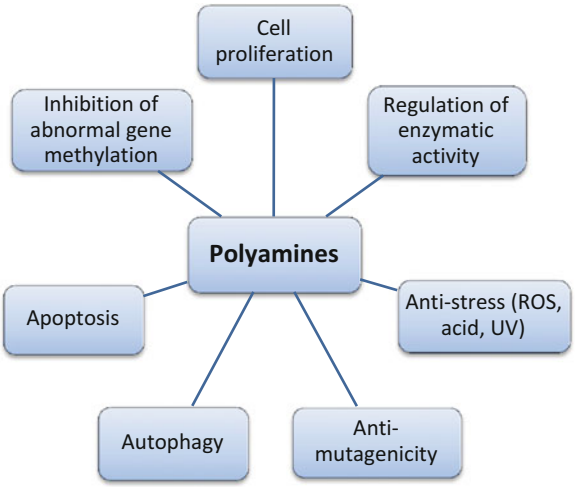


**Fig. 21.1** Polyamines preserve healthy cellular life. *ROS* reactive oxygen species

(Pillai and Shankel 1997). Soda et al. (2013) found that inhibition of age-associated and polyamine deficiency-induced abnormal gene methylation is a novel DNA protection mechanism of polyamines. It is notable that spermidine promotes autophagy, which is a conserved homeostatic process by which cells degrade and recycle cytoplasmic content and organelles using yeast, worms, flies, and human cultured immune cells (Eisenberg et al. 2009). However, it is unclear that the lifespan of mammalians is expanded by polyamine-induced autophagy. Some reports have suggested a link between polyamines and apoptosis; however, there has not yet been any conclusive evidence to prove this. For further details of effects of polyamines on apoptosis, several reviews have been published (Seiler and Raul 2005; Bjelaković et al. 2010). From these functions, polyamines ought to be recognized as a very important and uncommon bioactive substance that is involved in almost all biological phenomena in mammalian cellular life.

## 21.3 Polyamines and Mammalian Aging

If effects of polyamines on the preservation of healthy cellular life were to decline, what would happen? I believe that a decline in polyamine levels is a trigger of senescence and diseases. In fact, polyamine levels decrease with aging. Rat and mice studies have shown a decrease in ornithine decarboxylase (ODC) activity in the cerebral cortex, heart, and lungs with aging (Das and Kanungo 1982). This decline in polyamine levels with aging was observed in almost all tissues and was most notable in the dermal, cardiac, and muscular tissues (Nishimura et al. 2006). In a human study, Pucciarelli et al. determined the whole blood polyamine levels in 78 sex-matched unrelated individuals who were divided into three age groups: group 1 (31–56 years), group 2 (60–80 years), and group 3 (90–106 years) (Pucciarelli et al. 2012). The total polyamine content was significantly lower in groups 2 and 3 compared to group 1. In a related study (by the author), the amount of polyamines produced by intestinal microbiota in elderly individuals was found to be lower than that produced in healthy adults (Matsumoto and Benno 2007).

## 21.4 Inflammation and Polyamines

Polyamines, especially spermine, inhibit proinflammatory cytokine synthesis in human mononuclear cells (Zhang et al. 1997). Zhang et al. clarified the mechanism for the regulation of macrophage activation and inflammation by spermine as follows: in response to an inflammatory stimulus, the activity of the spermine uptake system in activated macrophages is increased, leading to the incorporation of spermine from the surrounding environment. Spermine accumulation at an inflammatory site enhances the intracellular macrophage uptake of spermine, resulting in the downregulation of macrophage activation and inflammation (Zhang et al. 1999).

Recently, suppression of inflammation on microglia, which are glial cells that function as the prime effector cell in the immune defense, and inflammatory responses in the central nervous system, were found to be induced by spermidine (Choi and Park 2012).

Expression of several adhesion molecules increases in an age-dependent manner. The adhesion and activation of immune cells mediate inflammation. Soda et al. established that lymphocyte function-associated antigen-1 (LFA-1) is an adhesion molecule and demonstrated that spermidine suppresses LFA-1 expression (Soda et al. 2005).

From these results, it can be concluded that polyamines have antiinflammatory activity through their actions on mononuclear cells and adhesion molecules.

## 21.5 Colon Health and Polyamines

The number of diseases that affect the colon is much higher than those that affect other organs. Moreover, colonic diseases are not comparable to diseases that affect other organs, because they are caused by intestinal bacteria that damage colonic tissue directly. Intestinal barrier function also declines as a consequence of aging (Ma et al. 1992). This phenomenon means that induction of inflammation is caused by the invasion of inflammatory factors derived from food and intestinal bacteria. Therefore, I believe that inhibition of colonic senescence may promote longevity.

Approximately $10^{14}$ bacteria, at least 160 different species, inhabit each individual's intestine and play a fundamentally important role in health and disease (Qin et al. 2010). Interestingly, almost all exogenous polyamines derived from meals are absorbed before they reach the lower parts of the intestine (Uda et al. 2003), whereas polyamines in the colon are synthesized by intestinal microbiota (Matsumoto et al. 2012).

Many studies have also been conducted on the relationship between polyamines and intestinal barrier function, including the maturation of postnatal intestinal tissue (Löser 2000), promotion of intestinal mucus and secretory immunoglobulin A secretion (Buts et al. 1994), recovery of injury (Lux et al. 1980), and induction of the synthesis and stability of tight junction-associated proteins, such as E-cadherin (Guo et al. 2003) and occludin (Guo et al. 2005). Briefly, intestinal polyamines are very important for protecting the physical invasion of inflammatory factors derived from food and bacteria from intestinal lumen. In response to aging, expression of a large number of genes involved in inflammation are enhanced and global hypomethylation levels are increased in the colon (Steegenga et al. 2012), which suggests that the suppression of inflammation and abnormal methylation that polyamines are able to induce might promote colonic health. Antimutagenic activity of polyamines may also prevent colon cancer. We demonstrated that upregulation of colonic polyamine concentrations by consumption of the probiotic *Bifidobacterium animalis* subsp. *lactis* LKM512 (hereafter referred to as LKM512) decreased levels of acute inflammation markers in hospitalized elderly patients, and also decreased mutagenic activity in hospitalized elderly patients (Matsumoto et al. 2001) and healthy adults (Matsumoto and Benno 2004).

## 21.6 Promotion of Longevity in Mammals Induced by Polyamines

The long-term oral intake of polyamine-rich diets increases blood polyamine levels in both mice and humans, although the intracellular de novo synthesis of the polyamines decreases with age and varies widely between individuals (Soda et al. 2009b). Soda et al. (2009a) investigated whether oral intake of polyamine-rich chow (spermine and spermidine) suppressed age-associated pathology in aged mice. An increase in polyamine concentration in the blood was only found in mice fed a high-polyamine chow at 50 weeks of age. Although the body weights of mice in all three groups were similar, the survival rate of mice fed high-polyamine chow was significantly higher than those in both low and moderate groups. Mice fed the high-polyamine chow analyzed at 88 weeks of age demonstrated lower incidence of glomerulosclerosis and increased expression of senescence marker protein-30 in the kidney and liver compared to those fed the low-polyamine chow.

On the other hand, we have been trying a novel approach to upregulate colonic luminal polyamines using probiotics based on the following hypothesis: increased polyamine concentration in the colonic tract represses senescence and promotes longevity by maintaining or restoring intestinal barrier function, or by exerting anti-inflammatory activity, antimutagenic activities, antioxidative properties, and autophagy (Matsumoto and Kurihara 2011). To test this hypothesis, we supplemented the diet of 10-month-old Crj:CD-1 female mice with LKM512 or spermine for 11 months, while the controls received no supplementation (Matsumoto et al. 2011). We found that LKM512-treated mice survived significantly longer than controls (Fig. 21.2a). The fecal concentrations of polyamines were significantly higher in LKM512-treated mice. Colonic mucosal tissue and function were also better in LKM512 mice, with increased mucous secretion from goblet cells and better maintenance of tight junctions (Fig. 21.3a). By analysis of gene expression levels of colon with reverse transcription-quantitative polymerase chain reaction (PCR) and

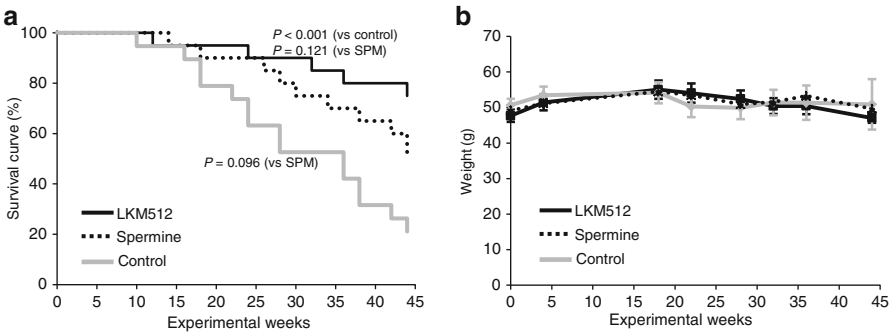


**Fig. 21.2** Impact of LKM512 on lifespan and weight (**a**); Kaplan–Meier survival curves (**b**). Weight differences between treatment groups during the study period

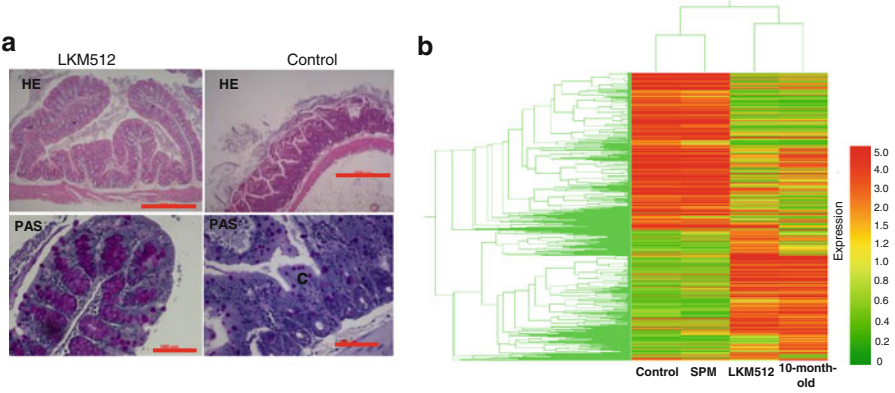


**Fig. 21.3** Influence of LKM512 administration on the colon. **a** Histology of proximal colon samples from mice treated with LKM512 (*left*) or phosphate-buffered saline (PBS) (control; *right*). The samples shown in the *upper panels* are stained with hematoxylin and eosin (H&E); those in the *bottom panels* are stained with periodic acid–Schiff (PAS). *Bars* H&E 500 m; PAS 100 m. **b** Microarray analysis of middle colon samples from LKM512-treated mice. Hierarchical clustering showing patterns of expression relationships among LKM512-treated, spermine-treated, control, and younger mice

DNA microarray, LKM512 administration was found to also downregulate the expression of aging-associated and inflammation-associated genes. Gene expression levels in 21-month-old LKM512-treated mice resembled those in 10-month-old untreated mice (Fig. 21.3b). In contrast, the oral administration of spermine did not improve longevity to the same extent as did the dose-intrinsic polyamines supplied by colonic bacteria altered by LKM512 administration (Fig. 21.2a). This difference may be related to the quantity of polyamines supplied: polyamines delivered orally are transient, but polyamines produced by colonic microbiota that have been enhanced by LKM512 treatment are continuously replaced. However, further studies are required to clarify the effective quantity of polyamines administered orally. Interestingly, there was no significant weight difference between the groups, or any noticeable difference in weight fluctuation during the study period, although mice in both treatment groups were given a standard pellet chow diet ad libitum (Fig. 21.2b). These results are similar to those obtained by Soda et al. (2009a). It is important to note that the longevity observed in mice given exogenous polyamines was not related to calorie restriction, which has been shown to increase longevity in a variety of species (Walford et al. 1987).

The mechanism behind the increased lifespan of intestinal luminal polyamine-upregulated mice is shown in Fig. 21.4. The polyamines produced induce the maintenance and/or recovery of intestinal barrier function by upregulating mucous secretion. Additionally, because of its antioxidative properties, it aids the prevention

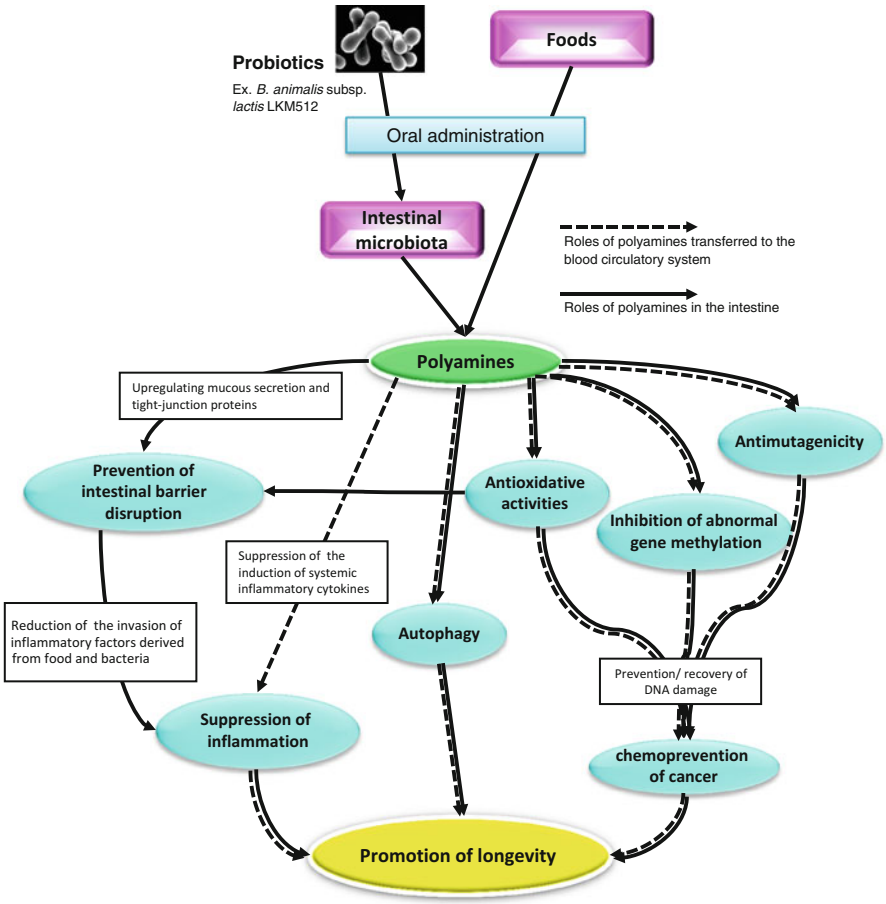


**Fig. 21.4** Mechanism underlying the increased lifespan of intestinal luminal polyamine-upregulated mice

of colonic barrier disruption. Maintenance of the intestinal barrier reduces the intestinal inflammatory activity of factors derived from food and bacteria. Moreover, increased levels of polyamines in the intestinal lumen lead to an increased polyamine concentration in the blood. These circulating polyamines suppress the induction and/or production of systemic inflammatory cytokines. At the same time, polyamines possess antimutagenic and antioxidative activities that prevent DNA damage. Thus, the effects of polyamines suppress chronic low-grade inflammation, thereby promoting longevity. Furthermore, autophagy induced by polyamines also promotes longevity.

## 21.7 Cancer and Polyamines

Since the discovery that polyamines were involved in the progression of cancer (Russell and Levy 1971), a large number of studies have investigated this relationship in more detail. This work has led to the tentative conclusion that polyamines are causative agents in cancer, a conclusion that was bolstered by the finding that progression of familial adenomatous polyposis and colorectal adenoma are prevented by concomitant administration of antiinflammatory agents and α-difluoromethylornithine (DFMO) (Gerner 2007; Thompson et al. 2010). However, most of the historical studies tested the effects of polyamines on existing tumors or on the growth of already established tumors (Sarhan et al. 1992; Cipolla et al. 2007). We have found no evidence that increased intake of polyamines promotes oncogenic transformation in normal cells and healthy animals. In spite of this evidence, we fully understand concerns that polyamines may act as a tumor promoter. Therefore, increasing polyamine levels by either exogenous supplementation or upregulation of intestinal luminal polyamine concentrations should be applied carefully to patients who already have cancer. Indeed, recent reports mention that polyamines actually function as cancer chemopreventive agents. For example, it was reported that adenosylmethionine decarboxylase 1, which is involved in spermidine biosynthesis, is a tumor suppressor using a human lymphoma model (Scuoppo et al. 2012). Soda et al. demonstrated that increased polyamine intake was associated with a decreased incidence of colon tumors induced by 1,2-dimethylhydrazine administration in BALB/c mice (Soda et al. 2013). Furthermore, there are many reports that polyamines are indispensable for homeostatic processes in a diverse group of organisms ranging from bacteria to plants and mammals. We note that polyamines are essential metabolic building blocks of life, because polyamines stimulate mRNA translation by altering mRNA structure in both prokaryotic and eukaryotic cells (Igarashi and Kashiwagi 2011) and spermidine is required for maturation of eIF5A (Park et al. 2010), an indispensable factor in protein synthesis. We hope that the results presented here encourage more researchers to investigate the diverse and important bioactivities of polyamines.

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# Chapter 22
# Polyamine Interactions with Plant Hormones: Crosstalk at Several Levels

**Raheel Anwar, Autar K. Mattoo, and Avtar K. Handa**

**Abstract** Polyamines play important roles in diverse plant growth and development processes, including seed germination, tissue lignification, organogenesis, flowering, pollination, embryogenesis, fruit development, ripening, abscission, senescence, and stress responses. In all these processes, synergistic and antagonistic interactions among various plant hormones have been shown. Although significant progress has been made in understanding the regulation of biosynthesis and signal transduction mechanisms for most of the plant hormones, inroads into the molecular mechanisms underlying polyamine (PA) action have just begun. Little is known or understood about the signal transduction pathways regulating a myriad of PA effects. We, therefore, embarked on evaluating and collating the information on changes in the transcriptome based on metabolic engineering of the PA pathway as well as mutants of PA biosynthesis. Also taken into consideration are the studies using exogenous application of PAs. Our analysis has revealed complex and differential relationships among the three main PAs—putrescine, spermidine and spermine—in regard to regulation of plant hormone biosynthesis and signaling. Putrescine was positively linked to the expression of genes regulating ABA biosynthesis but downregulated those of ethylene, jasmonates, and gibberellin biosynthesis, and the action of spermidine was found to be exactly opposite. Spermine, on the other hand, enhanced genes for ethylene and jasmonates biosynthesis but downregulated those for gibberellins and abscisic acid biosynthesis. In regard to hormone signaling pathways, spermidine positively regulated salicylic acid signaling genes, and those of auxin and cytokinins signaling genes were linked to spermine action. Putrescine was neutral to positive in regulating jasmonates signaling. PAs seem to be neutral in regard to brassinosteroids biosynthesis or signaling pathways.

**Keywords** ABA • Auxin • Biosynthesis • Brassinosteroids • Cytokinins • Ethylene • Gibberellins • Jasmonates • Salicylic acid • Signaling • Transcriptional factors

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## 22.1 Introduction

Polyamines (PAs) are biogenic amines with aliphatic polycationic properties and are ubiquitous in all living organisms. Although PAs were discovered more than 300 years ago (Vanleeuwenhoek 1978), it is only within the past few decades that significant progress has been made in understanding their role in plant growth and development (Bachrach 2010; Evans and Malmberg 1989; Galston and Sawhney 1990; Martin-Tanguy 2001; Nambeesan et al. 2008). PAs are essential for cell division and proliferation in all organisms and are implicated in diverse growth and development processes including chromatin function, protein synthesis, structural integrity of nucleic acids, and cellular membrane dynamics (Handa and Mattoo 2010; Kusano et al. 2008; Matthews 1993; Theiss et al. 2002; Thomas and Thomas 2001; Wallace 2009). Pharmacological evidence through exogenous application of PAs and recent molecular inroads through perturbation of endogenous PA levels by transgenic approach have demonstrated the important role of PAs in seed germination, tissue lignification, organogenesis, flowering, pollination, embryogenesis, fruit development, ripening, abscission, senescence, and stress responses (Alcazar et al. 2005; Gomez-Jimenez et al. 2010; Imai et al. 2004; Kusano et al. 2008; Mattoo et al. 2007; Minocha et al. 2014; Nambeesan et al. 2008; Takahashi and Kakehi 2010; Tisi et al. 2011; Urano et al. 2005). However, in spite of the myriad of effects of PAs confirmed in many organisms including plants, the molecular mechanisms involved are still poorly understood (Torrigiani et al. 2008).

The electrostatic or covalent binding of PAs to various macromolecules that causes conformational changes in chromatin, DNA, RNA, and protein structures and culminates in altered gene expression and physiological cellular responses led to the suggestion that they are important components of cellular proliferation (Thomas and Thomas 2001) and impact plant growth and developmental processes (Garufi et al. 2007; Kasukabe et al. 2004; Srivastava et al. 2007). Covalent binding of PAs to proteins, such as cationization and crosslinking, hypusine synthesis (a cofactor of eIF-5A), and accumulation of cytotoxic lipophilic PA derivatives may, in fact, lead to metabolic shifts in organisms (Seiler and Raul 2005; Takahashi and Kakehi 2010). Another property that is being researched is the ability of PAs, particularly spermine (Spm), to scavenge free radicals and thus impact reactive oxygen species (ROS) and redox signaling (Das and Misra 2004; Ha et al. 1998; Løvaas 1996). In plants, engineering the levels of spermidine (Spd) and spermine at the cost of putrescine (Put) was shown to affect glucose metabolism and carbon:nitrogen (C:N) signaling and alter cellular energy balance in fruits (Mattoo et al. 2006, 2007; Pirinen et al. 2007).

Suggestions for crosstalk among plant hormones and PAs emanated from studies in which many physiological and developmental processes were found synergistically or antagonistically modulated by PAs vis a vis plant hormones (Bitrián et al. 2012; Milhinhos and Miguel 2013). Biosynthesis of PAs, Spd and Spm, starts with the substrate, *S*-adenosylmethionine (SAM), which is also a substrate for the

plant hormone ethylene (Mattoo and White 1991). In situations when this substrate might become limiting, it could determine the outcome for which pathway, whether ethylene or Spd/Spm biosynthesis, would dominate (Harpaz-Saad et al. 2012; Lasanajak et al. 2014).

Another example of a hormone that appears to crosstalk with PAs is abscisic acid (ABA) in regulating abiotic stress responses, integrating ROS and nitric oxide (NO), and altering ion homeostasis, especially $Ca^{2+}$ (Alcazar et al. 2010). Neither a specific receptor nor a signal transduction mechanism is as yet discerned for PAs. Little is known or understood about the signal transduction pathways regulating a myriad of PA effects. We, therefore, analyzed and collated the information on changes in transcriptome obtained on transgenics where metabolic engineering of PA pathway was carried out as well as data on mutants of PA biosynthesis, and studies in which various PAs were exogenously applied to plant tissues. These analyses, summarized here, indicate that a complex network regulates interactions of both PAs and plant hormones.

## 22.2 Altered Endogenous Polyamine Levels Affect Transcriptome

Overexpression of yeast *S*-adenosylmethioinine decarboxylase (*ySAMDC*) under a fruit/ethylene-specific promoter E8 (*E8:ySAMDC*) resulted in two- to threefold increase in Spd and Spm content of ripening tomato fruit while lowering the concentration of Put to a minimum (Mehta et al. 2002). Comparison of the transcriptome of the wild-type (WT) and the *E8:ySAMDC* transgenic ripening tomato fruits revealed a massive change in the gene expression between the two genotypes, attributed to the increased levels of Spd and Spm in *E8:ySAMDC* lines (Srivastava et al. 2007). The metabolome of these genotypes showed corresponding changes as well (Mattoo et al. 2006). These data were compared for correlation analysis with changes in amino acids of poplar cells transformed with a constitutively expressed mouse *ODC* (*mODC*) gene that accumulated Put (Mohapatra et al. 2010). It was revealed that Put and Spd/Spm had mostly opposite effects, and therefore it was concluded that each PA may have a unique role in plant metabolome, as well as at the level of the transcriptome (Handa and Mattoo 2010; Mattoo and Handa 2008, Mattoo et al. 2010). Similarly, although Spd/Spm levels were positively correlated with changed transcript levels of early ripening genes, Put exhibited a negative correlation with transcript accumulation of many of the same genes (Handa and Mattoo 2010; Mattoo and Handa 2008; Mattoo et al. 2010); this was also true for several fruit quality attributes (Handa and Mattoo 2010). Evaluation of transcriptome changes associated with high Spd/Spm level, the *E8:ySAMDC* transgenic tomato, in comparison with the parental WT fruit (Mehta et al. 2002) have also been carried out using the TOM1 transcriptional microarray (Kolotilin et al. 2011). These investigators also found significant changes in gene expression patterns of *E8:ySAMDC* transgenic fruits compared to WT fruits as originally published (Srivastava et al. 2007). Effect of

exogenous application of Spd to mature green tomato fruit with or without exposure to increasing temperature stress on gene expression using the Affymetrix microarray also revealed upregulation of several genes related to defense responses, oxidation reduction, signal transduction, and hormone biosynthesis (Cheng et al. 2012).

Engineered constitutive expression of *Curubita ficifolia* Spd synthase (*SPDSYN*) in *Arabidopsis* plants also increased Spd and Spm content by about twofold. The transgenic plants had reduced chilling stress that correlated with enhanced expression of stress-responsive transcription factors and proteins (Kasukabe et al. 2004). Constitutive overexpression of arginine decarboxylase gene (*35S:AtADC2*) in *Arabidopsis* led to levels of Put in the transgenics more than 16 fold higher without significantly changing Spd and Spm contents (Alcazar et al. 2005). Accumulation of Put inhibited gibberellic acid (GA) biosynthesis, which resulted in dwarf stature and delayed flowering in transgenic *Arabidopsis* plants (Alcazar et al. 2005). Transcriptome analysis of *35S:AtADC2* and WT plants showed that overexpression of *ADC2* downregulated expression of dioxygenases genes (*GA20ox1*, *GA3ox1*, and *GA3ox3*), which are involved in the final step of GA metabolism, whereas transcription of genes involved in early steps of GA biosynthesis remained unaltered (Alcazar et al. 2005). Similarly, *Arabidopsis* plants constitutively overexpressing Spm synthase (*35S:AtSPMS-9*) had about threefold increase in Spm contents without perturbing Put and Spd levels in 15-day-old leaves (Gonzalez et al. 2011), an observation in agreement with results from a T-DNA insertion mutant of *Arabidopsis* in *SPMSYN* (*spms-2*) that showed about twofold decrease in Spm without alteration in Put and Spd contents (Gonzalez et al. 2011). Thus, it appears that each step in individual polyamine biosynthesis can dictate changes in their respective levels, with and without affecting the level of the other PAs. Spm levels in *35S:AtSPMS-9* transgenic *Arabidopsis* plants were positively correlated with plant resistance to *Pseudomonas viridiflava* (Gonzalez et al. 2011). Microarray analysis of transcriptional changes in the gene expression in *35S:AtSPMS-9* revealed that overproduction of Spm enhanced transcription of several transcription factors, kinases, nucleotide- and DNA/RNA-binding proteins, and the genes involved in pathogen perception and defense responses (Gonzalez et al. 2011). In another study, transcriptome profile of *SAMDC1*-overexpressing (*35S:AtSAMDC1*) was compared with those of *35S:AtADC2* and *35S:AtSPMS-9 Arabidopsis* plants to investigate role of polyamines in regulating abiotic stress pathways (Marco et al. 2011a, b). The Affymetrix ATH1 microarray-generated transcriptome showed that the functional enrichment of genes related to pathogen defense and abiotic stresses were commonly upregulated in Put- or Spm-accumulating *Arabidopsis* plants (Marco et al. 2011a, b). We further evaluated these global transcriptome profiles along with that obtained from Spd/Spm-accumulating tomato fruits (*E8:ySAMDC*) to delineate the roles of endogenous PAs levels on regulation of plant hormone biosynthesis and signaling pathway genes. Exogenous application of Spm also induced defense response in *Arabidopsis* against cucumber mosaic virus by modulating expression of genes involved in photorespiration, protein degradation, defense, protein folding, and secretion (Mitsuya et al. 2009).

## 22.3 Polyamine-Ethylene Crosstalk

PAs are considered as antisenescence growth regulators that seem antagonistic to ethylene-promoted leaf senescence, fruit ripening, and biotic stresses (Abeles et al. 1992; Alba et al. 2005; Alexander and Grierson 2002; Cheong et al. 2002; Evans and Malmberg 1989; Galston and Sawhney 1990; Giovannoni 2001; Klee 1993; Nambeesan et al. 2008; Tieman et al. 2000). Ethylene is involved in leaf epinasty, flower fading, abscission, fruit ripening, and senescence. Because SAM is the common substrate for ethylene, Spd and Spm biosynthesis, that exogenous application of PAs inhibits ethylene production in diverse plant tissues, and that ethylene inhibits activities of enzymes in PA biosynthesis pathway, a crosstalk among their biosynthesis pathways as well as during plant development was suggested (Apelbaum et al. 1981; Cassol and Mattoo 2003; Harpaz-Saad et al. 2012; Li et al. 1992; Mattoo and White 1991). However, the rate of ethylene production in E8:y*SAMDC* transgenic tomato fruits, that accumulated two- to threefold higher in Spd/Spm concentration, was much higher than the azygous control fruit (Mehta et al. 2002), which demonstrated that availability of SAM in vivo is not rate limiting for the biosynthesis of either ethylene or Spd/Spm and that both pathways could run simultaneously. Interestingly, in spite of higher ethylene production in the y*SAMDC*-overexpressing transgenic tomato fruit, a delay in on-vine ripening of transgenic fruits was observed. These results indicated a dominant role of Spd/Spm over ethylene during the fruit-ripening process. This inference was also supported by investigations on another genetic event that involved overexpression of yeast spermidine synthase gene (*SPDSYN*), driven also by E8 promoter (Nambeesan et al. 2010). The *E8:ySPDSYN* fruit also had increased Spd as well as ethylene, and yet had extended shelf life, lower shriveling rate, and delayed decay compared to WT tomato fruits.

The high endogenous Spd/Spm concentrations in the *E8:ySAMDC* tomato fruit were accompanied by about twofold increase in *ACS* transcripts compared to WT fruits (Mattoo et al. 2007). The *35S:AtSPMS-9* transgenic *Arabidopsis* had also increased *ACS* transcript levels in the leaves (Gonzalez et al. 2011), but *ACS6* transcripts were about threefold downregulated in the Put accumulating leaves of *35S:AtADC2* transgenic *Arabidopsis* leaves (Alcazar et al. 2005). Reduction in *ACS* transcripts in *35S:AtADC2 Arabidopsis* leaves and increase in *E8:ySAMDC* tomato fruits and *35S:AtSPMS-9 Arabidopsis* leaves (Alcazar et al. 2005; Gonzalez et al. 2011; Kolotilin et al. 2011; Mattoo et al. 2007) support the contrasting roles played by Put and Spd/Spm in plant growth and development, as proposed earlier (Handa and Mattoo 2010; Mattoo et al. 2010). However, more data from other transgenic plants and PA mutants are needed to unequivocally prove this hypothesis.

The microarray data obtained from plant tissues with altered PA levels support the contention that the biogenic amines alter ethylene response by modulating expression of ethylene signaling pathway components (Fig. 22.1). Transcriptome data indicate a potential of PAs to increase ethylene production by enhancing expression of *ACS*. The expression of *S-Adenosylmethionine Synthetase* (*MAT*) and *Mitogen-Activated Protein Kinase Kinase* (*MAPKK*) were also upregulated in high

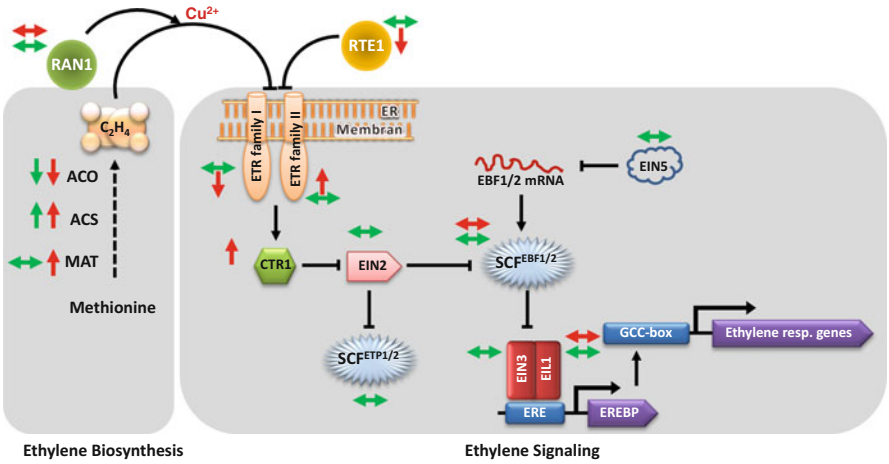


**Fig. 22.1** Consensus effects of Spd and Spm on ethylene metabolism and signaling cascade. *Green* (*Arabidopsis* leaves) or *red* (tomato fruit) *vertical arrows* indicate up- and downregulated gene transcripts by Spd/spm. *Black arrows* and *blunt heads* indicate stimulatory or inhibitory effects, respectively. *ACO* 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, *ACS* ACC synthase, *CTR1* constitutive triple response 1, *EBF1* EIN3-binding F-box protein 1, *EBF2* EIN3-binding F-box protein 2, *EIL1* ethylene-insensitive3-like 1, *EIN2* ethylene insensitive 2, *EIN3* ethylene insensitive 3, *EIN5* ethylene insensitive 5, *ERE* ethylene response element, *EREBP* ethylene response element-binding protein (including ERFs), *ETP1* EIN2 targeting protein 1, *ETP2* EIN2 targeting protein 2, *ETR* ethylene receptor, *MAT* *S*-adenosylmethionine synthetase, *RAN1* responsive-to-antagonist 1, *RTE1* reversion-to-ethylene sensitivity 1

*Spd/Spm* accumulating transgenic tomato fruit (Kolotilin et al. 2011). It is now known that the ethylene response is negatively regulated by its receptors. In the absence of ethylene, ethylene receptors (ETRs, ERS, EIN4) interact with ER-localized family of Raf-like serine/threonine kinases (CTRs, constitutive triple response factors) and suppress the ethylene signaling cascade (Zhong et al. 2008). Five ethylene receptors and one CTR1 have been identified in *Arabidopsis*, whereas six ethylene receptors and three CTRs are present in the tomato genome (Chen et al. 2010; Klee and Tieman 2002). As shown in Fig. 22.1, responsive-to-antagonist1 (RAN1) delivers $Cu^{+}$ cofactor to ethylene receptor to make the multiprotein complex functional (Hirayama et al. 1999; Woeste and Kieber 2000). Constitutive overexpression of tomato *Green-ripe* (*GR*) or an *Arabidopsis Reversion-To-Ethylene Sensitivity 1* (*RTE1*) homologue reduced ethylene responsiveness while its mutant exhibited weaker ethylene insensitivity in tomato fruit, indicating that, similar to receptors and CTRs, repression of GR is also required to perceive ethylene action in tomato (Barry and Giovannoni 2006). To activate signaling pathway, ethylene binds to its receptors, causing conformation change and inactivation of CTRs; this releases downstream signal transducer ethylene insensitive 2 (EIN2) from suppression (Ju et al. 2012) and induces transcription of EIN3 and EIN3-like transcription factors (EILs) (Ji and Guo 2013). EIN3 and EILs differentially regulate transcription of

ethylene response factors (ERFs) that regulate transcription of ethylene-responsive target genes (Guo and Ecker 2004). The high endogenous levels of Put or Spm had no effect on the transcript levels of either ethylene receptors or *CTR1* in *Arabidopsis* leaves (Alcazar et al. 2005; Gonzalez et al. 2011). However, higher endogenous Spd/Spm levels in *E8:ySAMDC* tomato fruits correlated with downregulation of ethylene receptors *ETR2* and *ETR3* (*NEVER-RIPE*), but *ETR4* and *ETR6* were upregulated (Kolotilin et al. 2011). *CTR* homologues were also either upregulated by two- to threefold or remained unaltered in *E8:ySAMDC* tomato fruits (Kolotilin et al. 2011).

Based on the absence or presence of putative transmembrane domains, ethylene receptors have been broadly divided into subfamilies I and II, respectively (Hall et al. 2007). Tomato ETR2 and ETR3 belong to subfamily I and ETR4 and ETR6 belong to subfamily II (Binder 2008). Increase in the expression of *ETR4*, *ETR6*, and *CTRs* in Spd/Spm-accumulating fruits is analogous to increase in the abundance of receptor–CTR complexes that would strengthen the negative regulation of ethylene. This analogy would also explain why, in spite of higher ethylene production in *ySAMDC* and *ySPDSYN* transgenic tomato fruits, the ethylene responsiveness manifested as fruit ripening was reduced in these fruit (Mehta et al. 2002; Nambeesan et al. 2010). It is possible that the higher expression of subfamily II receptors is also a compensatory response to decreased *ETR3* expression (Tieman et al. 2000).

The transcriptome changes in response to exogenously applied PAs support the afore-discussed results that PAs alter expression of ethylene signaling pathway genes. Application of 1 mM Put or Spd to abscising mature olive fruit increased transcript levels of ethylene receptor *ERS1*, even though *CTR1* expression showed slight decrease and abrogated the ethylene signaling pathway (Parra-Lobato and Gomez-Jimenez 2011). Exogenous application of 0.1 mM Spd to peach fruit mesocarp lowered ethylene production and led to higher transcript levels of *ETR1* and *ERS1* (Ziosi et al. 2006). Peach fruits treated with 1 mM Spd had reduced ethylene perception with enhanced expression of subfamily II receptors and CTRs transcripts, which is indicative of PAs nexus with ethylene signaling (Torrigiani et al. 2012). In the absence of ethylene, EIN3-regulating F-box proteins (EBF1 and EBF2) degrade EIN3 by the ubiquitin/proteasome pathway (Guo and Ecker 2003). Silencing of *EBF1* and *EBF2* has been reported to cause constitutive ethylene response phenotype in tomato (Yang et al. 2010). However, the *E8:ySAMDC* tomato fruit pericarp was neutral for the expression of *EBF1* (Kolotilin et al. 2011). Nonetheless, grapes treated with guazatine, an inhibitor of PA oxidase, accumulated Put and were upregulated in *EIN3* and *EBF2* expression (Agudelo-Romero et al. 2014).

Taken together these results indicate a very complex crosstalk between PAs and various components of ethylene action. The complexity is partly driven by the presence of a family of genes for both the biosynthesis and action of ethylene, each under a complex developmental and environmental regulation. Homologues of various gene families not only are redundant in some cases and take over the function of their counterpart, but also their transcription can compensate the loss/inexpression of a family member (Tieman et al. 2000).

## 22.4 Polyamine-Jasmonate Crosstalk

Jasmonates (JAs), mainly derived from linolenic acid, are oxylipin signaling molecules that regulate a wide range of plant developmental and growth processes including male fertility, root growth, tendril coiling, fruit ripening, and inducing plant defense responses (Farmer and Ryan 1990; Srivastava and Handa 2005; Wasternack 2007; Yan et al. 2007). The early enzymatic reactions catalyzed by plastid-localized lipoxygenases (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) yield *cis*-(+)-12-oxophytodienoic acid (OPDA). Conversion of OPDA to JA in peroxisomes is catalyzed in series by 12-oxophytodienoate reductase 3 (OPR3), acyl-CoA oxidase (ACX), multifunctional protein (MFP), *L-3-ketoacyl*-CoA thiolase (KAT), and acyl-thioesterase (ACH) (Creelman and Mullet 1997; Schaller and Stintzi 2009; Wasternack 2007). Jasmonoyl-*L*-isoleucine synthetase (JAR) and JA carboxyl methyltransferase (MJT) catalyze the conversion of JA to jasmonate-isoleucine (JA-Ile) and methyl ester of JA (MeJA), respectively (Fonseca et al. 2009). The JAs-induced changes in PA biosynthesis seem to delay fruit ripening (Yoshikawa et al. 2007; Ziosi et al. 2007, 2009), stimulate tolerance against pathogens and insect herbivores (Kaur et al. 2010; Walters et al. 2002), induce wound response (Pérez-Amador et al. 2002), and decrease low-temperature injuries (Wang and Buta 1994; Yoshikawa et al. 2007). JAs have also been implicated in the production of conjugated PAs in vegetative plant tissue (Keinänen et al. 2001; Tebayashi et al. 2007; Zhang et al. 2007).

MeJA was reported to upregulate expression of *ADC*, *ODC*, and *SAMDC*, increase oxidation and conjugation of PAs, and inhibit shoot formation in tobacco thin layers (Biondi et al. 2001). Either MeJA application or mutations imparting constitutive JA signaling resulted in increased production of caffeoyl-Put in tomato leaves, whereas *jasmonate insensitive1* (*jai1-1*) and *coi1* mutants defective in JA perception were inhibited in the production of caffeoyl-Put in tomato and tobacco leaves, respectively (Chen et al. 2006; Paschold et al. 2007). That MeJA induces conjugation of PAs and involves R2R3-MYB8, a JA responsive transcription factor, was revealed through RNAi-mediated silencing of R2R3-MYB8 (Kaur et al. 2010). In these transgenic tobacco plants, downregulation of *SPDSYN* was accompanied by lack of phenylpropanoid-PA conjugates, caffeoyl-Put and dicaffeoyl-Spd (Kaur et al. 2010; Onkokesung et al. 2012). Transcription of Put *N*-methyltransferase (PMT), an enzyme that converts Put into *N*-methyl-Put, is stimulated by MeJA (Shoji et al. 2000) and inhibited by herbivore-induced ethylene production (Winz and Baldwin 2001). It has been discovered that a TA-rich region and a GCC motif in the promoter of tobacco *PMT* regulate MeJA-induced transcription of *PMT* (Xu and Timko 2004). In fruit tissues, however, MeJA induced accumulation of free PAs without altering the levels of conjugated PAs. Application of *n*-propyl dihydro jasmonate (PDJ), a synthetic derivative of MeJA, enhanced accumulation of free PAs by 30–60 % in epicarp and mesocarp of peach fruit, whereas MeJA application had negligible effects on PA and ethylene production. In both treatments, PCA-soluble and PCA-insoluble PAs could not be detected in peach fruits (Ziosi et al.

2009). Cosuppression of *SlLOXB* resulted in 60–90 % reduction in MeJA and 50 % reduction in free PAs in transgenic compared to WT tomato fruits (Kausch et al. 2012). Increase in free Spd and Spm in MeJA-treated fruits correlates with low-temperature stress tolerance in zucchini squash (Wang and Buta 1994), mango (González-Aguilar et al. 2000), and apples (Yoshikawa et al. 2007), suggesting a role of free Spd and Spm in fruit ripening and low-temperature stress tolerance.

Little is known about the effects of PAs on production, conjugation, perception, and signal transduction of JAs. Overexpression of y*SAMDC* intensified accumulation of ω-3 fatty acids in ripening tomato fruit, with α-linolenic acid (C18:3 n3) levels increasing to more than 50 % of total fatty acids (Kolotilin et al. 2011). High Spd/Spm also increased transcript levels of *LOX* and *3-Keto-Acyl-Coenzyme A Thiolase (KAT)* in tomato fruit (Kolotilin et al. 2011; Srivastava et al. 2007). Constitutively overexpressed *ySAMDC* caused a 24- to 90-fold increase in *LOX* transcripts whereas expression of spermine synthase gene *SPMSYN* (*35S:AtSPMS-9*) in *Arabidopsis* increased the levels of *LOX*, *AOC3*, and *OPR3* transcripts (Gonzalez et al. 2011; Marco et al. 2011a). In addition to upregulation of JA biosynthesis genes, transcript levels of JA-conjugating sulfotransferases (ST2A, ST2B) were also increased in *35S:AtSPM-9 Arabidopsis* plants, suggesting that higher PAs also stimulate conjugation of JAs (Gonzalez et al. 2011). The overaccumulation of Put in *ADC2* transgenic *Arabidopsis* plants showed about threefold downregulation of the *LOX* gene, an effect opposite to that of Spd and Spm (Alcazar et al. 2005).

Studies using an *Arabidopsis* mutant (*coi1*) resistant to coronatine, a phytotoxin structurally similar to jasmonate, have helped our understanding of the JAs signaling pathway in plants (Santner and Estelle 2009). Binding of jasmonate-isoleucine (JA-Ile) to COI1, a F-box protein that forms E3 ubiquitin ligase complex ($SCF^{COI1}$), promotes binding to JAZ proteins and facilitates their degradation (Fig. 22.2); this liberates JIN1/MYC2 from repression, activating the jasmonate response (Chini et al. 2009; Sheard et al. 2010; Thines et al. 2007; Yan et al. 2007). MYC proteins are transcription factors that belong to group IIIe of the bHLH family. MYC2/JIN1 binds with conserved G-box *cis*-elements in the promoter region of target transcriptional activators or repressors to regulate JA-induced gene expression (Fig. 22.2) (Fonseca et al. 2009; Kazan and Manners 2008; Pauwels and Goossens 2011; Turner et al. 2002). Transcript levels of genes encoding JAZ proteins were upregulated by higher Spd/Spm in transgenic tomato fruits and *Arabidopsis* leaves, but downregulated in Put-accumulating *35S:AtADC2 Arabidopsis* leaves (Alcazar et al. 2005). However, expression of neither *JAR1* nor *COI1* changed in *35S:AtSPMS-9* or *spms-2 Arabidopsis* leaves (Gonzalez et al. 2011). It is noted here that the JAZ proteins repress EIN3 and EIL1 in the ethylene signaling pathway, suggesting that PAs can inhibit ethylene signaling by positively regulating transcription of JAZ proteins (Pauwels and Goossens 2011). JERF3 is a two-way acting regulatory hub induced by JA, ethylene, ABA, salt, and cold, and binds with both dehydration-responsive element DRE and GCC-box to regulate expression of genes in multiple defense mechanisms (Wang et al. 2004). The report that *JERF3* transcripts were downregulated in *E8:ySAMDC* transgenic tomato supports an inhibitory role of PAs on jasmonate and ethylene signal transduction pathways (Kolotilin et al. 2011).

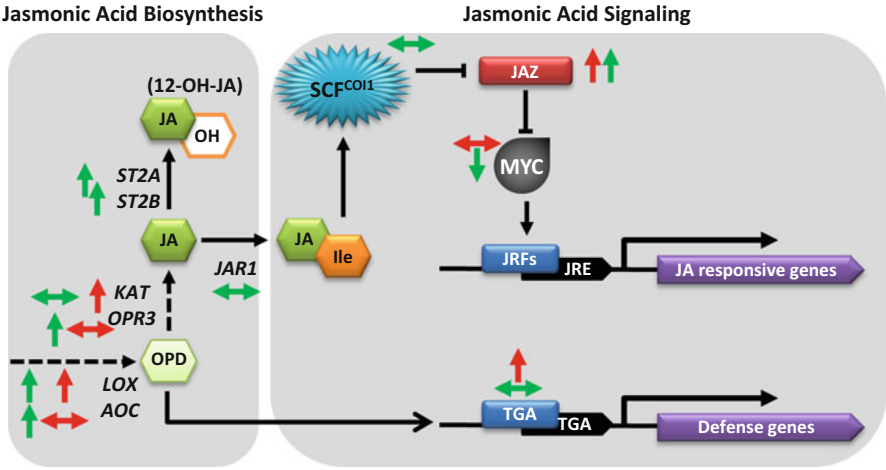


**Fig. 22.2** Consensus effects of Spd and Spm on jasmonic acid metabolism and signaling cascade. Description same as in legend to Fig. 22.1. *12-OH-JA* 12-hydoxyjasmonic acid, *AOC* allene oxide cyclase, *COI1* coronatine insensitive 1, *JA-Ile* jasmonate (JA)-isoleucine conjugate, *JAR1* jasmonate resistant 1, *JAZ* jasmonate-zim-domain protein, *JRE* jasmonate response element, *JRFS* jasmonate response factors, *KAT* 3-keto-acyl-coenzyme A thiolase, *LOX* lipoxygenase, *MYC and TGA* transcription factor proteins, *OPDA cis*-(+)-12-oxophytodienoic acid, *OPR3* oxophytodienoate reductase 3, *ST2A* and *ST2B* sulfotransferase

OPDA induces plant resistance against wounding and pathogen infection by activating expression of genes via a COI1-independent signaling pathway dependent on TGA transcription factors (Mueller et al. 2008; Stintzi et al. 2001). OPDA-specific response genes (ORGs) that were strongly upregulated by OPDA but less so by JA or MeJA have been identified (Taki et al. 2005). Among the OPDA-responsive genes differentially transcribed in *35S:AtSPMS-9 Arabidopsis* leaves, expression of 29 genes was positively correlated with *SPMSYN* expression (Gonzalez et al. 2011) whereas 17 OPDA-specific genes were downregulated in the Put-accumulating *35S:AtADC2 Arabidopsis* leaves (Alcazar et al. 2005). OPDA-regulated TGA transcription factors bind to the TGA motif in promoter regions to enhance expression of defense response genes (Zhang et al. 1999). Increased transcript levels of *TGA* transcription factors in *E8:ySAMDC* transgenic tomato fruits that accumulate high Spd/Spm suggest that PAs might have a role in OPDA-mediated signal transduction in tomato fruit (Kolotilin et al. 2011).

## 22.5 Polyamine-Auxin Crosstalk

Auxins are well-known plant growth hormones involved in plant processes including embryogenesis, apical patterning, stem elongation, development of vascular tissues, and root initiations that mediate gravitropic and phototropic responses in

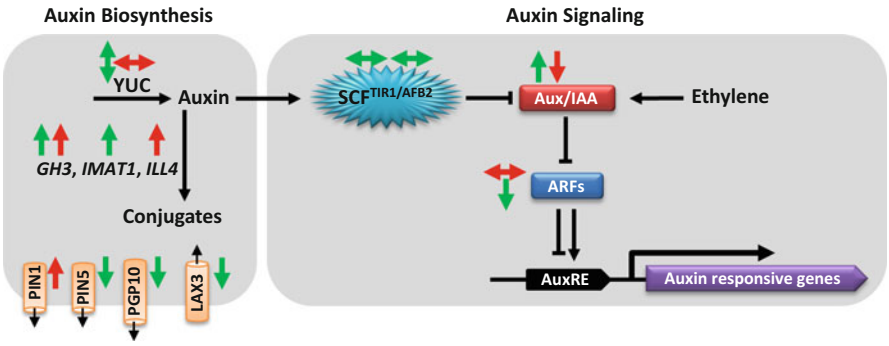


**Fig. 22.3** Consensus effects of Spd and Spm on auxin metabolism and signaling cascade. Description is same as in legends to Fig. 22.1. *AFB2* auxin signaling F-box protein 2, *ARFs* auxin response factors, *Aux/IAA* indole-3-acetic acid inducible (members of Aux/IAA protein family), *AuxRE* auxin response *cis*-elements, *GH3* IAA-amido synthetase, *IAMT1* IAA carboxyl methyl transferase 1, *ILL4* IAA amidohydrolase, *LAX3* like AUX1 3, *PGP10* P-glycoprotein 10 (auxin efflux carrier), *PIN1* PIN-formed 1, *PIN5* PIN-formed 5, *TIR1* transport inhibitor response 1, *YUC* YUCCA

plants (Chapman and Estelle 2009; Vanneste and Friml 2009). Auxin concentration and distribution in plant tissues is regulated by its biosynthesis, conjugation, modification, and transport (Korasick et al. 2013). Indole-3-acetic acid (IAA) is a predominant bioactive form of auxin derived from tryptophan, mainly through tryptophan aminotransferase and YUCCA/flavin monooxygenase pathways. The auxin signaling pathway is a complex, with Aux/IAA proteins being the major responsive proteins that negatively regulate auxin signaling by associating with carboxyl-terminal dimerization domain of auxin response factors (ARFs) and repressing their transcriptional activity (Fig. 22.3) (Chapman and Estelle 2009; Vanneste and Friml 2009). Auxin binds and activates F-box receptor transport inhibitor response 1 (TIR1) and TIR1-related auxin signaling F-box (AFB) proteins. The activated TIR1 and AFB proteins, as a part of ubiquitin E3 ligase complex ($SCF^{TIR1/AFB}$), interact with Aux/IAA and direct them to 26S proteasome-mediated degradation (Chapman and Estelle 2009; Vanneste and Friml 2009). This event releases ARFs transcription factors from repression and thus depresses or activates transcription of auxin response genes (Chapman and Estelle 2009). ARFs are *trans-acting* factors that bind with TGTCTC-containing auxin-response *cis*-elements (AuxREs) within the promoter region of auxin-regulated genes (Hagen and Guilfoyle 2002). Among hundreds of gene regulated by auxin, major classes of auxin early response genes, in addition to *Aux/IAA*, include *Small Auxin Up RNA* (*SAUR*) and *GH3* proteins (Hagen and Guilfoyle 2002). Until now, at least 34 *Aux/IAA,* 23 *ARFs*, and 20 *GH3* genes have been identified in *Arabidopsis* (Hagen and Guilfoyle 2002), and 36 *Aux/IAA* and 17 *ARFs* have been identified in tomato (Audran-Delalande et al. 2012; Kumar et al. 2011; Wu et al. 2012b).

The nexus of polyamines with auxin came about through transgenic research and new information through transcriptomic analysis. The overexpression of *SPMSYN* (*35S:AtSPMS-9)* in *Arabidopsis* leaves resulted in differential expression of the family of YUCCA genes: more than twofold decrease in the expression of *YUC1* and *YUC10* occurred and a two- to threefold increase in *YUC5* and *YUC8* (Gonzalez et al. 2011). However, higher PA levels in *E8:ySAMDC* tomato fruit did not alter steady-state transcript levels of *YUC-like flavin monooxygenase* and *ToFZY*, a putative orthologue of *YUC4* and *FLOOZY (FZY)* (Expósito-Rodríguez et al. 2007; Kolotilin et al. 2011). Also, application of Spd did not induce accumulation of free and bound levels of IAA in radish seedlings that were either unstressed or stressed with copper (Choudhary et al. 2012b) or chromium (Choudhary et al. 2012a). *SPMSYN* transgenic leaves had twice as many transcripts of *IAA2*, *IAA3*, *IAA6*, *IAA19*, *IAA20*, *IAA29*, and *IAA33* whereas transcripts for *ARF6*, *ARF8*, and *ARF13* were actually downregulated as compared to the control WT plants (Gonzalez et al. 2011). It is noted here that, in contrast to these data, levels of *IAA3, IAA6*, and *IAA25* transcripts were lower in *E8:ySAMDC* transgenic tomato fruit.

Distribution of auxin and establishment of its gradient within tissues is crucial for some of the auxin-regulated plant growth and development processes (Ikeda et al. 2009; Tivendale et al. 2014; van Berkel et al. 2013). Several auxin influx and efflux carriers have been characterized that regulate cell-to-cell transport of auxin. Auxin influx into the cell is either passive or regulated by AUX1/LAX (auxin permease/like AUX) family of $H^+$ symporters (Pattison and Catala 2012; Peret et al. 2012). Auxin efflux carriers have been classified into two major families. The PIN (PIN-FORMED) family of auxin efflux carriers is plant-specific and regulates polar auxin transport. MDR/PGP (multi-drug resistance/P-glycoprotein) family of auxin exporters belongs to the ATP-binding cassette (ABCB) superfamily of transporters that ubiquitously handle distribution of various molecules and nutrients (Zazimalova et al. 2010). How PAs affect auxin transport has not yet been studied. Ectopic expression of *35S:AtSAMDC1* or *35S:AtSPMS*-9 in *Arabidopsis* downregulated two auxin efflux carriers, *PGP10* and *PIN5,* and one auxin influx carrier *LAX3* (Gonzalez et al. 2011; Marco et al. 2011a). Decrease in Spm in *spms-2* mutant leaves also downregulated *PIN1*, *PIN7*, and *ABCB4* (Gonzalez et al. 2011). On the other hand, *ADC2*-overexpression upregulated *ABCB4* by fourfold in *Arabidopsis* leaves. However, it needs to be noted that *Arabidopsis* overexpressing *SPMSYN* had threefold increase in free Spm levels over the controls whereas the *spms-2* mutant, deficient in Spm, had twofold lower free Spm content than its WT (Gonzalez et al. 2011). Suppression of xylem vessel differentiation is also a unique characteristic of a structural isomer of Spm, thermoSpm (Kakehi et al. 2008; Muñiz et al. 2008; Takano et al. 2012). Higher Spm levels in *35S:AtSPMS-9* enhanced expression of *ACL5*, a thermoSpm-encoding gene, by more than eightfold (Gonzalez et al. 2011). ThermoSpm and auxin act antagonistically to fine tune the temporal and spatial pattern of xylem differentiation (Yoshimoto et al. 2012). Increase in the expression of *ACL5* further explains the mechanism behind downregulation of auxin in *Arabidopsis* leaves. Increase in *ABCB4* in the Put-accumulating line also adds to the hypothesis that functions of Spd and Spm are opposite to that of Put (Handa and Mattoo 2010;

Mattoo et al. 2010). In contrast to downregulation of auxin carriers in *Arabidopsis* leaves, transcript levels of *PIN1-type* proteins were upregulated by twofold in *E8:ySAMDC* tomato fruit (Kolotilin et al. 2011). It appears that the interaction of PAs with auxin in plant development may, in fact, be tissue specific.

Members of the *SAUR* family are plant specific and constitute a major set of auxin-responsive genes (Wu et al. 2012a). In *35S:AtSPMS-9 Arabidopsis* leaves, most of the *SAUR* genes including *SAUR8*, *SAUR11*, *SAUR35*, *SAUR36*, *SAUR37*, *SAUR38*, *SAUR51*, and *SAUR56* were downregulated. In parallel, Spm deficiency in *spms-2* leaves downregulated *SAUR21* (Gonzalez et al. 2011). The *ySAMDC-tomato* fruit pericarp was twice richer than the controls in the transcripts for SAUR protein genes, *SAUR1*, *SAUR36*, and solyc06g053290 (Kolotilin et al. 2011). *Arabidopsis* and tomato contain more than 72 and 98 *SAUR* genes, respectively. Although some members in the SAUR family have been implicated in hypocotyl elongation during shade avoidance (Roig-Villanova et al. 2007), plant response to high temperature (Franklin et al. 2011), and cell expansion and tropic responses (Spartz et al. 2012), most *SAUR* genes have not yet been functionally characterized, possibly because *SAUR* mRNA and proteins are unstable, short lived (Gil and Green 1996; Knauss et al. 2003; Newman et al. 1993; Zenser et al. 2003), and do not bear similarity with any motif of known biochemical function (Spartz et al. 2012).

Spm inhibits expression of several auxin carriers, *Aux/IAA*, *ARF*, and *SAUR* genes, in *Arabidopsis* (Gonzalez et al. 2011), whereas higher Spd and Spm levels enhance expression of some of the auxin-regulated genes in tomato fruit (Kolotilin et al. 2011). Higher Put in *Arabidopsis* leaves upregulates *GH3.4*, *GH3.6*, and *GH3.17* transcripts and overaccumulation of Spm upregulates *GH3.3* and *GH3.5*, *and IAMT1*, an IAA carboxyl methyltransferase that catalyzes the methylation of IAA (Li et al. 2008). In *E8:ySAMDC* tomato fruit, higher Spd/Spm also increased *GH3.8* and *IAA-AMINO ACID HYDROLASE 4* (*ILL4*) transcripts (Kolotilin et al. 2011). Further characterization of the role of PAs in regulating auxin function is needed to shed more light on the role of PAs in the auxin biology.

## 22.6 Polyamine-Gibberellins Crosstalk

Gibberellins (GAs) are tetracyclic, diterpenoid carboxylic acids that regulate many cellular processes in plants including seed dormancy breakdown, stem and root elongation, trichome development, leaf expansion, pollen maturation, flowering, sex expression, fruit setting, parthenocarpic fruit development, and fruit ripening (Itoh et al. 2008). As many as 136 GA compounds have been identified in plants, fungi, and bacteria. GA biosynthesis is regulated by various internal and external stimuli such as auxin, brassinosteroids, cytokinins, light, stratification, salinity, and cold (Hedden and Thomas 2012). Terminal steps in GA biosynthesis are catalyzed by two key enzymes: GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) (Fig. 22.4). These enzymes belong to the 2-oxoglutarate (2OG) and Fe(II)dependent oxygenase superfamily (Hedden and Thomas 2012).

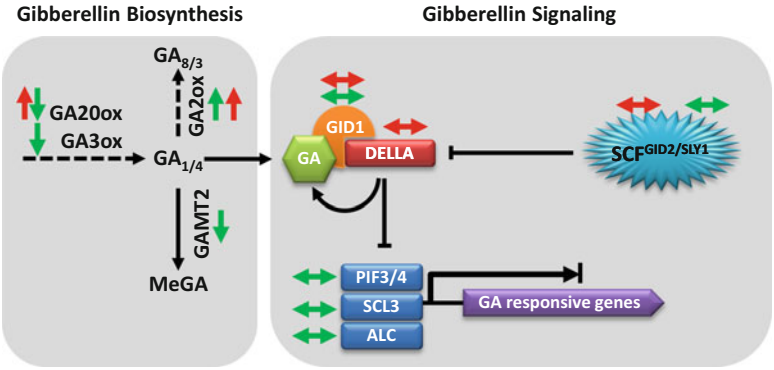


**Fig. 22.4** Consensus effects of Spd and Spm on gibberellin metabolism and signaling cascade. Description is same as in legends to Fig. 22.1. *ALC* alcatraz, *GA* gibberellin, *GA2ox* GA 2-oxidase, *GA20ox* GA 20-oxidase, *GA3ox* GA 3-oxidase, *GAMT2* gibberellic acid methyltransferase 2, *GID1* GA insensitive dwarf 1, *GID2* gibberellin insensitive dwarf 2, *MeGA* methyl ester form of GA, *PIF3* phytochrome interacting factor 3, *PIF4* phytochrome interacting factor 4, *SCL3* scarecrow-like 3, *SLY1* Sleepy 1

The connection between PAs and GA arose serendipitously during characterization of transgenic *Arabidopsis* plants that ectopically expressed arginine decarboxylase (*35S:AtADC2*) and accumulated high amounts of free and conjugated Put (Alcazar et al. 2005). These transgenic plants were dwarf with delayed flowering and exhibited reduced expression of *GA3ox3* and *GA20ox1* as well as reduced production of $GA_1$ (Alcazar et al. 2005). The accumulation of Spm in *35S:AtSPMS-9 Arabidopsis* leaves also correlated with downregulation of *GA 20-oxidases* and *GA 3-oxidase* transcripts; in addition, GA catabolism was enhanced by upregulation of *GA 2-oxidases* transcripts (Gonzalez et al. 2011). Downregulation of *gibberellic acid methyltransferase 2* (*GAMT2*), an enzyme that converts active $GA_1$ and $GA_4$ into its methyl ester forms, MeGAs, was observed in *35S:AtSPMS-9 Arabidopsis* leaves (Gonzalez et al. 2011). On the other hand, *E8:ySAMDC* tomato fruit was upregulated in the transcript levels for both the GA 20-oxidase and GA 2-oxidases during fruit ripening (Kolotilin et al. 2011), which suggests that Spd/Spm promote GAs conjugation and their conversion into inactive forms. Accumulation or deficiency of Put or Spd/Spm has not thus far been found to alter expression of any GA singling gene in transgenic *Arabidopsis* leaves or tomato fruits (Fig. 22.4).

## 22.7 Polyamine-Cytokinin Crosstalk

Another plant hormone with important function in growth and development as well as in environmental responses is a group called cytokinins (CKs). CKs are involved in processes such as seed development, tuber formation, shoot and meristem

development, chloroplast biogenesis, vascular differentiation, leaf expansion, leaf senescence, nutrient balance, and stress tolerance (Hwang et al. 2012; Sakakibara et al. 2006). CKs and PAs mutually regulate several common physiological and developmental processes as tested by physiological-pharmacological approaches (Galston 1983). The reports that both CKs and PAs generate nitric oxide (NO), an intra- and intercellular gaseous messenger involved in regulation of biotic and abiotic stress responses, has attracted some attention (Wimalasekera and Scherer 2009). Although CKs have been reported as potential inducers of NO, whether NO regulates the CK signaling pathway is yet to be determined (Romanov et al. 2008). Similarly, Spd/Spm but not Put induce production of NO (Moreau et al. 2010; Tun et al. 2006), but any potential links between PAs and NO are yet to be verified (Yamasaki and Cohen 2006).

CKs enhance ADC activity and Put accumulation in excised cucumber cotyledons in culture (Suresh et al. 1978), rice embryos (Choudhuri and Ghosh 1982), and etiolated pea seedlings (Palavan et al. 1984). Treatment of etiolated cucumber cotyledons with kinetin increased polyamine oxidase (PAO) activity, decreased SAMDC activity along with a decrease in Spd levels, and increased Put content (Sobieszczuk-Nowicka et al. 2007). CK treatment of lettuce cotyledons, dark-grown cucumber cotyledons (Walker et al. 1988), and soybean suspension cultures (Mader and Hanke 1997) induced accumulation of free Put, but not free Spd and Spm (Cho 1983). However, higher Put levels were not required for CK-induced greening of cucumber cotyledons (Walker et al. 1988). Earlier, Put and CK were reported to act synergistically during embryogenesis, but increase in Spd and Spm levels seemed to play an important role in embryo development and plantlet formation in celery (Danin et al. 1993). CK enhanced free Put and reduced Spd and Spm levels during expansion of excised cucumber cotyledons and gametophore bud formation in moss (Legocka and Zarnowska 2002). Interestingly, deficiency of CKs does not affect Put biosynthesis, but reduction in *trans*-zeatin, an active form of cytokinin, was accompanied by increases in free Put, Spd, and Spm during and after germination of maple seedlings (Walker et al. 1989). In kinetin-treated moss, decrease in Spd was also accompanied by increase in PCA-insoluble levels of all three PAs (Legocka and Zarnowska 2002).

The loss-of-function of *SAMDC4* in *bud2-2 Arabidopsis* mutant led to an increase in Put (11.5 %) with a corresponding decrease in Spd (9.3 %) and Spm (13.3 %) and exhibited altered root and shoot architecture (Cui et al. 2010; Ge et al. 2006). In another study, exogenous Spm prevented expression of *ARR5* in *Arabidopsis* (Romanov et al. 2002). However, no change in transcript levels of CK biosynthetic genes was found in *Arabidopsis* leaves expressing either *35S:AtADC2*, *35S:AtSPMS-9* or *35S:AtSAMDC1* or in tomato fruits expressing *E8:ySAMDC* (Alcazar et al. 2005; Gonzalez et al. 2011; Kolotilin et al. 2011; Marco et al. 2011a).

CK dehydrogenase (CKX, previously known as cytokinin oxidase) cleaves the CK side chain, producing aldehydes and adenine derivatives. The transcripts of CKX genes were found elevated for *CKX1* and *CKX6* in *35S:AtSPMS-9*, for *CKX6* in *35S:AtSAMDC1 Arabidopsis* leaves (Gonzalez et al. 2011; Marco et al. 2011a), and for *CKX2* in *E8:ySAMDC* tomatoes (Kolotilin et al. 2011), suggesting roles for PAs in CK degradation and homeostasis. Zeatin-*O*-glucosyltransferase, which

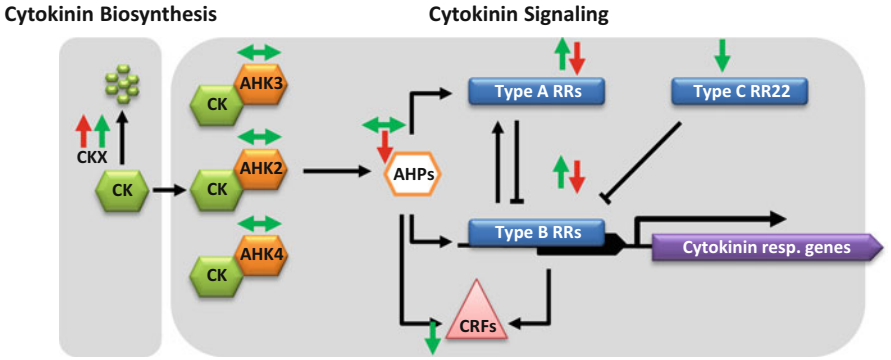


**Fig. 22.5** Consensus effects of Spd and Spm on cytokinin metabolism and signaling cascade. Description is same as in legends to Fig. 22.1. *AHK2* histidine kinase receptor 2, *AHK3* histidine kinase receptor 3, *AHK4* histidine kinase receptor 4, *AHPs* histidine-containing phosphotransfer protein, *RRs* response regulators, *CK* cytokinin, *CKX* cytokinin dehydrogenase, *CRFs* cytokinin response (*resp.*) factors

glucosylates *trans*-zeatin into *trans*-zeatin-*O*-glucoside, was downregulated in *E8:ySAMDC* tomatoes (Kolotilin et al. 2011). In contrast, *CKX* transcripts remained unaffected in *35S:AtADC2 Arabidopsis* plants, suggesting that Put may have a limited role in CK biosynthesis (Alcazar et al. 2005).

The canonical CK signaling cascade in *Arabidopsis* includes three histidine kinase receptors: AHK2, AHK3, and AHK4/CRE1/WOL (Hwang et al. 2012) (Fig. 22.5). CK triggers autophosphorylation of conserved histidine residue in cytoplasmic kinase domain of AHKs. Histidine-containing phosphotransfer proteins (AHPs) accept a phosphoryl group via aspartic acid residue on the AHK receiver domain and transfer it to an aspartic acid residue on the N-terminal receiver domain of type-B response regulators (RRs). Phosphorylation of type-B RRs releases repression of their C-terminal DNA-binding domain and activates type-A RRs CK response factors (CRFs) and other response genes. As a negative feedback regulation, Type-A RRs negatively regulate phosphorelay circuitry (Argueso et al. 2010; Hwang et al. 2012). In the absence of CK, AHK4 dephosphorylates AHPs to inhibit phosphorelay in signaling cascade (Mähönen et al. 2006). F-box KISS ME DEADLY (KMD) family proteins negatively interact with type-B RRs and target them for SCF/proteasome-mediated degradation (Kim et al. 2012, 2013).

How do transgenic plants engineered for PA levels fare in terms of the CK signaling cascade just described? In *E8:ySAMDC* tomato fruits, response regulators *AHP1* protein, type-A (*TRR3*) and type-B (*RR8*), were downregulated, supporting an antagonistic effect of higher PAs on CK signaling (Kolotilin et al. 2011). On the other hand, *35S:AtSPMS-9 Arabidopsis* leaves were downregulated for *CRF8* transcripts but type-B (*ARR1*) and type-A response regulators (*ARR3*, *5*, and *7*) were upregulated (Gonzalez et al. 2011). A type-C response regulator *ARR22* was downregulated in Spm-accumulating transgenic *Arabidopsis* leaves. Further confirmation

of these results would help establish if the Spm and Spd are involved in the cytokinin signaling pathway. At present it seems that CKs favor Put biosynthesis and inhibit Spd and Spm accumulation by stimulating ADC and PAO activities and also possibly by increasing their conjugation. Also, Put does not appear to affect CK biosynthesis or signaling whereas Spd and Spm do so by stimulating the catabolism of CK by upregulating CK dehydrogenases. However, Spd inhibits whereas Spm promotes expression of genes involved in CK signaling.

## 22.8 Polyamine-Abscisic Acid Crosstalk

Abscisic acid (ABA), a sesquiterpenoid, is another plant hormone involved in the development and stress responses of plants, which include seed germination, lateral root formation, leaf and fruit size development, and stomatal closure in response to drought stress (Raghavendra et al. 2010). The first committed, rate-limiting step in ABA biosynthesis is the cleavage of plastid-localized 9-*cis*-violaxanthin and 9-*cis*-neoxanthin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin (Handa et al. 2014 and references therein). Short-chain dehydrogenase reductase catalyzes the conversion of xanthoxin into abscisic aldehyde, which is converted into ABA by abscisic acid aldehyde oxidase (AAO). ABA is catabolized by ABA-8′-hydroxylases into 8′-OH-ABA, followed by isomerization to phaseic acid. Additionally, glucosyltransferase glucosylates ABA to its storage and transport form.

Changes in ABA and PA levels have been studied during seed maturation and germination, and fruit growth and ripening, as well as in response to low-temperature, water-stressed, and UV-B conditions to understand interactions between them (Antolín et al. 2008; Bagni et al. 1980; Cvikrová et al. 1998; Fromm 1997; Gomez-Jimenez et al. 2001; Hurng et al. 1994; Lee et al. 1995b; Martinez-Madrid et al. 1996, 2002; Nayyar et al. 2005; Puga-Hermida et al. 2003; Rakitin et al. 2008; Serrano et al. 1995; Valero et al. 1998; Yokota et al. 1994; Yoshikawa et al. 2007). Under water stress conditions, transcript levels of *ADC*, *SPDSYN*, and *SPMSYN* were upregulated in *Arabidopsis* and impaired in ABA-insensitive (*abi1-1*) and ABA-deficient (*aba2-3*) *Arabidopsis* mutants (Alcazar et al. 2006a). *ADC2* transcripts and activity were reduced in *NCED3* knockout *Arabidopsis* mutant *nc3-2* (Urano et al. 2009). ABA treatment stimulated expression of *SAM1* and *SAM3*, encoding *S*-adenosyl-L-methionine synthetase, in tomato (Espartero et al. 1994), *SPDS3* transcripts in *Arabidopsis* (Hanzawa et al. 2002), and enhanced Put and Spm, but not Spd, levels in drought-tolerant cv. *Populus popularis* compared to drought-susceptible cv. Italica (Chen et al. 2002). ABA and stressors such as high salt and dehydration upregulated expression of *AtADC2* leading to accumulation of Put, a decrease in Spd, and unaltered Spm levels (Urano et al. 2003). The chilling-tolerant rice seedlings had increased Put but not Spd and Spm levels upon chilling (Lee et al. 1997). Thus, both ABA and PUT have been implicated in reducing plant damage from low-temperature stress in tomato plants (Jiang et al. 2012). In wheat, the ABA treatment enhanced Put and Spm but decreased Spd (Kovacs et al. 2010;

Rakitin et al. 2009). In leaf discs from drought-tolerant grapevine, ABA enhanced expression of PA synthesizing (*ADC*, *ODC*, and *SAMDC*) as well as catabolizing enzymes (DAO and PAO) resulting in a net two- to fourfold accumulation of Put and Spm but reduction in Spd levels, whereas leaf discs from drought-susceptible grapevine were higher in only DAO and PAO transcripts (Toumi et al. 2010). Presence of ABA-response elements (ABREs) in promoter regions of *ADC2*, *SAMDC1*, *SAMDC2*, *SPDS1*, and *SPMSYN* in *Arabidopsis* suggest that ABA-response elements may regulate PA biosynthesis (Alcazar et al. 2006b).

Reciprocal complementation tests on *adc* (Put-deficient) and *aba2-3* (ABA-defective) mutants suggested that Put positively regulates expression of *NCED3* in response to cold stress (Cuevas et al. 2008, 2009). High Put level in transgenic *35S:AtADC2 Arabidopsis* downregulated the CYP707A gene that encodes ABA-catabolizing enzyme ABA-8′-hydroxylase (Alcazar et al. 2005). Together, these add to findings mentioned earlier in that Put and ABA positively regulate each other's biosynthesis under abiotic stress (Alcazar et al. 2010). However, Spd and Spm act antagonistically to Put and inhibit ABA synthesis and induce its hydroxylation. For example, Spm reduced polyethylene glycol (PEG)-induced osmotic stress by modifying ABA and antioxidant levels in soybean pods and seeds (Radhakrishnan and Lee 2013a, b); Spm accumulation in *35S:AtSPM-9* and *35S:AtSAMDC1 Arabidopsis* leaves inhibited expression of *ALDEDHYE OXIDASE* (*AAO2*); and enhanced levels of Spd and Spm in transgenic *E8:ySAMDC* tomato fruits, decreased *AAO4* transcripts and increased in abscisic acid 8-hydroxylase 3 transcripts. Application of Spd did not alter free or bound titers of ABA in radish seedlings under normal growth condition (Choudhary et al. 2012b) or under chromium stress (Choudhary et al. 2012a), but reduced copper-induced accumulation of bound ABA in radish seedlings (Choudhary et al. 2012b).

The ABA signaling network is well characterized and has been elegantly described elsewhere (Cutler et al. 2010; Raghavendra et al. 2010; Umezawa et al. 2010; Weiner et al. 2010). ABA binds to cytosol- and nucleus-localized pyrabactin resistance (PYR)/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR) family of ABA receptors, causing inhibition of protein phosphatase 2Cs (PP2Cs), the negative regulators of ABA signaling. Upon release from PP2C-mediated repression, the SNF1-related kinase 2 (SnRK2) phosphorylates bZIP-like transcription factors including ABA-insensitive 5 (ABI5) and ABA-responsive element binding factor 2 (ABF2/AREB1) and ABF4/AREB2 (Shukla and Mattoo 2008). After phosphorylation these factors bind with *cis*-regulatory ABA-response elements (ABRE, ACGTGT) and regulate expression of abiotic stress-responsive genes (Fig. 22.6). SnRK2 also phosphorylates and regulates activity of other target proteins including anion channel slow anion channel-associated 1 (SLAC1), inward rectifying $K^+$ channel In *Arabidopsis thaliana* (KAT1), and reactive oxygen species (ROS)-producing respiratory burst oxidase homologue F (RbohF). The constitutive expression of *NtPYL4* receptor resulted in a 2.6-fold increase in total PAs in tobacco hairy roots compared to control roots (Lackman et al. 2011), but higher Spm levels in *35S:AtSPMS-9 Arabidopsis* leaves did not show any specific trend in regulation of *PYL* genes (Gonzalez et al. 2011). Accumulation of higher PAs in *35S:AtSPMS-9*

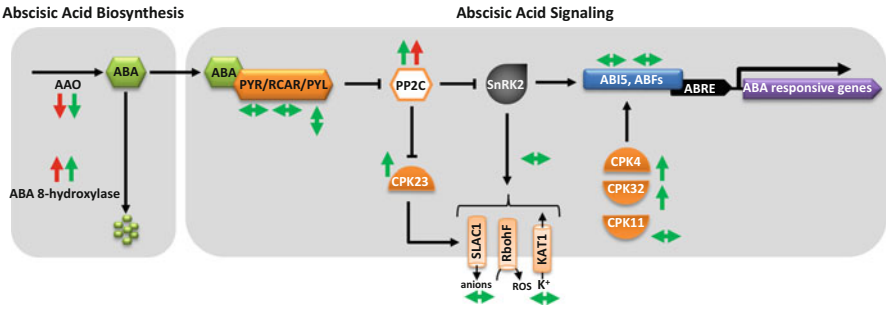


**Fig. 22.6** Consensus effects of Spd and Spm on abscisic acid metabolism and signaling cascade. Description is same as in legends to Fig. 22.1. *AAO* abscisic acid aldehyde oxidase, *ABA* abscisic acid, *ABFs* abscisic acid responsive element-binding factors, *ABI5* abscisic acid insensitive 5, *ABRE* ABA response element, *CPK4* calcium-dependent protein kinase 4, *CPK11* calcium-dependent protein kinase 11, *CPK23* calcium-dependent protein kinase 23, *CPK32* calcium-dependent protein kinase 32, *KAT1* $K^+$ channel in *Arabidopsis thaliana*, *PP2C* protein phosphatase 2C, *PYL* PYR1-like, *PYR* pyrabactin resistance, *RCAR* regulatory component of ABA receptor, *RbohF* ROS-producing respiratory burst oxidase homologue F, *SLAC1* slow anion channel-associated 1, *SnRK2* SNF1-related kinase 2

*Arabidopsis* leaves or *E8:ySAMDC* tomato fruits resulted in two- to threefold increase in transcript levels of *PP2C* proteins, suggesting that higher PAs inhibit both ABA accumulation and ABA signaling by altering expression of PP2C proteins (Gonzalez et al. 2011; Kolotilin et al. 2011). Put was shown to inhibit ABA degradation but ABA signaling genes in *35S:AtADC2 Arabidopsis* leaves were not altered, suggesting that Put may not play a major role in ABA signaling (Alcazar et al. 2005).

## 22.9 Polyamine-Salicylic Acid Crosstalk

Salicylic acid (SA) is an immunity signaling molecule in plant response to pathogens (Rojo et al. 2003; van Wees et al. 2000), low temperature (Lei et al. 2010; Zhang et al. 2013), high temperature (Kaplan et al. 2004; Widiastuti et al. 2013), high salinity (Jayakannan et al. 2013; Mutlu and Atici 2013; Singh and Gautam 2013; Tufail et al. 2013), heavy metals (Idrees et al. 2013; Mostofa and Fujita 2013; Pandey et al. 2013), and water deficit (de Agostini et al. 2013; Marcinska et al. 2013). SA biosynthesis is derived via the shikimate pathway intermediate chorismate, which is ultimately converted into SA via either the isochorismate pathway or phenylalanine pathway. However, under stress conditions, plants synthesize SA mainly from the chloroplast-localized isochorismate pathway (Dempsey et al. 2011). Several enzymes in plants have been characterized that catalyze modification of SA through glycosylation, methylation, amino acid conjugation, or hydroxylation (Dempsey et al. 2011; Garcion and Métraux 2007; Lee et al. 1995a; Sendon et al. 2011; Verpoorte and Memelink 2002).

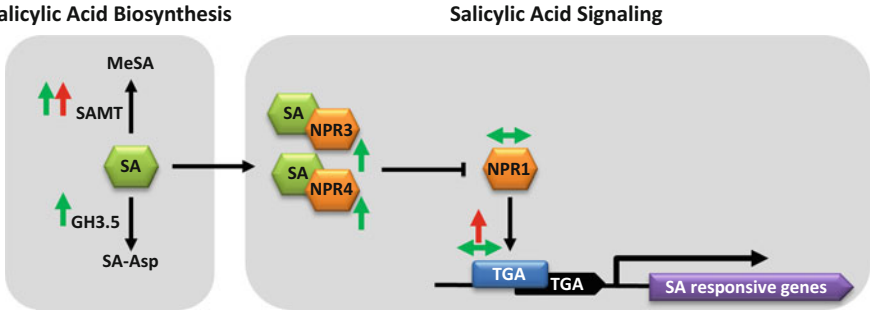


**Fig. 22.7** Consensus effects of Spd and Spm on salicyclic acid metabolism and signaling cascade. Description is same as in legends to Fig. 22.1. *GH3.5* GH3-like phytohormone amino acid synthetase, *MeSA* methyl salicylate, *NPR1* non-expressor of pathogenesis-related gene 1, *NPR3* NPR1-like protein 3, *NPR4* NPR1-like protein 4, *SA* salicylic acid, *SA-Asp* Salicyloyl-L-aspartic acid, *SAMT* SAM-dependent carboxyl methyltransferase, *TGA* TGA transcription factor

Exogenous application of SA was shown to enhance accumulation of free Put, Spd, and Spm in *Arabidopsis*, maize leaves, tomato fruits, bamboo shoots, asparagus, citrus, and callus cultures of carrots (Luo et al. 2012; Sudha and Ravishankar 2003; Wei et al. 2011; Zhang et al. 2011; Zheng and Zhang 2004). Spd also enhanced accumulation of 2-*O*-β-D-glucosyl salicylic acid, a conjugated form of SA in TMV-resistant tobacco plants (Lazzarato et al. 2009). Transgenic *Arabidopsis* plants (*35S:AtSPMS-9*, *35S:AtSAMDC1*) or tomato fruits (*E8:ySAMDC*) with enhanced Spd/Spm levels showed about twofold or higher increase in transcript levels of SAM-dependent carboxyl methyltransferase (*SAMT*), an enzyme that catalyzes methylation of SA into methyl salicylate (MeSA). The *35S:AtSPMS-9 Arabidopsis* leaves had higher transcripts of GH3-like phytohormone amino acid synthetase (*GH3.5*), an enzyme that catalyzes conjugation of SA to aspartic acid, producing salicyloyl-*L*-aspartic acid (SA-Asp). The tobacco leaf disc specifically responded to Spm by enhancing salicylic acid-induced protein kinase (SIPK, an orthologue of MPK6) and wound-induced protein kinase (WIPK) via mitochondrial dysfunction (Mitsuya et al. 2009; Takahashi et al. 2003). The SIPK, WIPK, and their homologues have been implicated in defense signaling against osmotic stress (Droillard et al. 2000; Hoyos and Zhang 2000; Ichimura et al. 2000; Mikołajczyk et al. 2000), low temperature (Ichimura et al. 2000; Jonak et al. 1996), ozone treatment (Samuel et al. 2000), wounding (Bogre et al. 1997; Ichimura et al. 2000), and pathogen invasion (Cardinale et al. 2000; Desikan et al. 2001; Ligterink et al. 1997; Nühse et al. 2000; Zhang and Klessig 1998). Collectively, these results indicate mutual positive regulation of SA conjugates and PAs biosynthesis.

Non-expressor of pathogenesis-related gene 1 (NPR1) is an ankryin-repeat-containing protein that interacts with TGA transcription factors to promote transcription of SA-responsive defense genes (Wu et al. 2012c). NPR3 and NPR4, paralogues of NPR1, are CUL3 ligase adaptors and function as SA receptors (Fu et al. 2012). Under physiological conditions, NPR3 and NP4 stimulate NPR1 degradation to inhibit SA signal transduction (Fig. 22.7). Pathogen infection induces SA binding

with NPR3 and NPR4 in a concentration-dependent manner and releases NPR1 from repression (Kaltdorf and Naseem 2013). Spm-accumulating *Arabidopsis* plants (*35S:AtSPMS-9*) had twofold higher levels of both *NPR3* and *NPR4,* suggesting it inhibits SA signaling by enhancing degradation of NPR1 by CUL3-NPR3 and CUL3-NPR4. Although both SA and Spm have been reported to enhance plant tolerance against pathogens (Raju et al. 2009), their crosstalk during plant–pathogen interactions remains to be determined.

Spd-induced response against infection seems to be mediated by salicylic acid (Lazzarato et al. 2009), whereas a response elicited by Spm seems independent of SA signaling pathway (Hiraga et al. 2000; Mitsuya et al. 2007; Takahashi et al. 2003, 2004; Uehara et al. 2005; Yamakawa et al. 1998). Put may not regulate SA biosynthesis or signaling, as was ascertained by studies showing that Put-accumulating *Arabidopsis* plants (*35S:AtADC2*) did not show any differential expression in SA biosynthetic or signaling genes.

## 22.10 Polyamine-Brassinosteroid Crosstalk

Brassinosteroids (BRs) are C27, C28, and C29 steroidal hormones that, in recent years, have been associated with plant cell elongation, vascular differentiation, root growth, responses to light, resistance to stresses, and senescence (Clouse and Sasse 1998; Kim and Wang 2010). Their biosynthetic pathways were first elucidated using cultured *Catharanthus roseus* cells, but identification of genes and enzymatic characterization have been intensively investigated using the *dwarf* mutants defective in BR biosynthesis or perception (Fujioka and Yokota 2003). More than 50 naturally occurring BRs have been identified. BRs are derived from membrane-associated sterol campesterol through multiple C-6 oxidation steps whereas campesterol is synthesized mainly from isopentenyl disphosphate (Bajguz 2012; Choudhary et al. 2012c; Fujioka and Yokota 2003; Shimada et al. 2001). BRs have been shown to play essential roles in a wide range of physiological and developmental processes and now considered as plant hormones (Kim and Wang 2010). Exogenous applications of PAs or 24-epibrassinolide (EBL, an active form of BRs) have been implicated in enhancing stress tolerance against drought, chilling, and salt stresses in rice, tomato, and *Adiantum capillus-veneris* plants, respectively (Farooq et al. 2010; Jiang et al. 2012; Sharma et al. 2014). EBL enhanced accumulation of Put but inhibited production of higher PAs (Spd/Spm) in radish seedling with or without Cu or Cr stress, and supplementing EBL with Put further enhanced accumulation of Put and Spd (Choudhary et al. 2011). However, enhanced PAs in *Arabidopsis* plants (*35S:AtADC2*, *35S:AtSAMDC1*, *35S:AtSPMS-9*) and tomato fruits (*E8:ySAMDC*) were not found to have corresponding changes in any of the known BR biosynthesis gene transcripts.

The brassinosteroid signal transduction pathway is given in Fig. 22.8. In *Arabidopsis*, BR binds with brassinosteroid insensitive 1 (BRI1) receptor and induces its autophosphorylation and dissociation from BRI1 kinase inhibitor (BKI1). Active BRI1 forms a heterodimer with BRI1-sssociated receptor kinase 1 (BAK1) and phosphorylates BR-signaling kinase 1 (BSK1) and constitutive differential

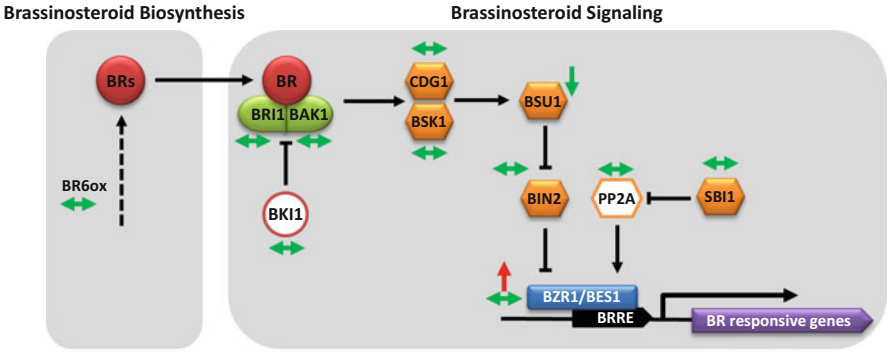


**Fig. 22.8** Consensus effects of Spd and Spm on brassinosteroid metabolism and signaling cascade. Description is same as in legends to Fig. 22.1. *BAK1* BRI1-associated receptor kinase 1, *BES1* BRI1-EMS suppressor 1/BZR2, *BIN2* brassinosteroid insensitive 2, *BKI1* BRI1 kinase inhibitor, *BR* brassinosteroid, *BR6ox* BR-6-oxidase, *BRI1* brassinosteroid insensitive 1, *BRRE* brassinosteroid response element, *BSK1* BR-signaling kinase 1, *BSU1* BRI1-suppressor 1, *BZR1* brassinazolr resistant 1, *CDG1* constitutive differential growth 1, *PP2A* protein phosphatase 2A, *SBI1* suppressor of BR1

growth 1 (CDG1) that, in turn, phosphorylates BRI1-suppressor 1 (BSU1). BSU1 deactivates brassinosteroid insensitive 2 (BIN2) through dephosphorylation, releasing brassinazole resistant 1 (BZR1) and BRI1-EMS suppressor 1 (BES1, also named BZR2) from repression. BZR1 and BES1 then regulate transcription of BR-responsive genes either directly or through transcription factors. BZR1 and BES1 are also activated by protein phosphatase 2A (PP2A), which is itself negatively regulated by suppressor of BR1 (SBI1) via methylation (Choudhary et al. 2012c; He et al. 2005; Kim and Wang 2010; Zhu et al. 2013). Transgenic *Arabidopsis* plants deficient in Spm (*spms-2*) showed 1.6-fold upregulation of BSU1 transcripts whereas tomato fruits with higher Spd and Spm showed 2.9-fold upregulation of BES1/BZR1 homologue protein 2 (Solyc02g063010) at breaker stage and then 2.5-fold decrease thereafter at red stage. Transcript levels of genes encoding BR signal transduction proteins remained unaltered in PA-accumulating transgenic *Arabidopsis* plants (*35S:AtADC2*, *35S:AtSAMDC1*, *35S:AtSPMS-9*). More studies are needed for deeper insight in our understanding of the role(s) of PAs in BR biosynthesis and/or signaling.

## 22.11 Concluding Remarks and Perspective

Plants are sessile organisms that have acquired the ability to adapt to a continuously changing environment. A wealth of information has emerged during the twentieth century showing that plants accomplish growth and propagation of their species by activating specific signaling pathways which allow them to survive under optimal to harsh environmental conditions. Perception of the environmental clues is largely

controlled by a few plant hormones: auxin, cytokinin, gibberellins, abscisic acid, ethylene, salicylic acid, brassinosteroids, and jasmonates. However, it is now recognized that the plant response to environmental conditions is complex and orchestrated by a network of the equally complex crosstalk among plant hormones (Garay-Arroyo et al. 2012). PAs are emerging as important plant growth and development regulators (Handa and Mattoo 2010). They play essential roles in both physiological and developmental processes (Nambeesan et al. 2008). We recently proposed that PAs act as 'rejuvenator molecules' and restore to an aging cell the metabolism of the younger cell in plants (Handa and Mattoo 2010; Mattoo et al. 2010). Spd extends the shelf life of tomato fruit and retards seasonal senescence of plants (Nambeesan et al. 2010) is an observation in tune with a similar role proposed for Spd in other organisms (Eisenberg et al. 2009). Although many plant cellular processes are also regulated by one or another plant hormones, little is understood about crosstalk among plant hormones and biogenic amines. As collated here, the transcriptomic data suggest a complex relationship among the three PAs and their role(s) in the biosynthesis and signaling pathways of plant hormones. However, these analyses only provide an initial evaluation of the interactions among PAs and other plant hormones, based on a limited number of genes and plant systems analyzed. Our models provide a simplistic road map that can be further modified and revised as more transcriptome, proteome, and metabolome data become available.

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# Part X
# Diagnosis and Drug Targets

# Chapter 23
# $N^1$,$N^{12}$-Diacetylspermine in Human Urine: Performance as a Tumor Marker, Quantification, Production, and Excretion

**Masao Kawakita, Kyoko Hiramatsu, Shun-suke Moriya, Keijiro Samejima, and Kei-ichi Takahashi**

**Abstract** $N^1$,$N^{12}$-Diacetylspermine (DiAcSpm) is a minor polyamine component of human urine. DiAcSpm levels are frequently elevated in the urine of patients suffering from various cancers, including colorectal, breast, urogenital, and lung cancer. DiAcSpm is an attractive candidate for use as a tumor marker because elevated levels of urinary DiAcSpm occur more frequently in the early stages of cancers compared to other tumor markers. To facilitate the clinical application of DiAcSpm in cancer diagnosis, simple procedures using highly DiAcSpm-specific antibodies have been developed, including a kit based on colloidal gold aggregation adapted for use with an automatic biochemical analyzer and an immunochromatographic device intended for personal use. Recent studies have shown that DiAcSpm levels increase in cancer tissues early in the course of cancer development and are excreted in the urine without being reabsorbed from the renal tubules: this explains why urinary DiAcSpm levels are elevated in patients with early-stage cancers.

**Keywords** APAO • Biomarker • Diacetylspermine • Early cancer detection • SSAT • Tumor marker • Urine

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## 23.1 The Major Human Urinary Polyamines and $N^1$,$N^{12}$-Diacetylspermine

Acetylputrescine and $N^1$- and $N^8$-acetylspermidine account for more than 90 % of polyamines excreted in human urine. $N^1$,$N^{12}$-diacetylspermine (DiAcSpm) typically accounts for approximately 0.5 % of total human urinary polyamine content (Hiramatsu et al. 1995). DiAcSpm in urine from healthy individuals was first detected by high performance liquid chromatography (HPLC) analysis using an enzymatic detection system consisting of immobilized acetylpolyamine amidohydrolase, polyamine oxidase and putrescine oxidase (Hiramatsu et al. 1994, 1995). Subsequent analyses revealed that the level of DiAcSpm is frequently increased in urine from patients with urogenital malignancies compared to urine from healthy individuals (Sugimoto et al. 1995).

In 1971, long before DiAcSpm was identified in human urine, Russell (Russell 1971) reported that the amount of polyamines excreted in urine was higher in patients with cancer than in healthy individuals. Subsequently, an extensive polyamine analysis was carried out to answer the question of whether the level of polyamines in urine could serve as an indicator for malignant diseases, but the results obtained were, on the whole, rather disappointing (Bachrach 1992). The performance of urinary polyamines (only free and monoacetylated polyamines were known at that time) as clinical markers were found to be unsuitable for clinical use because there were too many false negatives and false positives, partly because of large individual variations in polyamine excretion and because benign disorders were often associated with considerable increases in urinary polyamine levels. In contrast, DiAcSpm seemed promising because the fraction of false negatives was much smaller than that of other polyamine components (Sugimoto et al. 1995).

## 23.2 Characteristics of Urinary DiAcSpm in Healthy Adults

The amounts of DiAcSpm and creatinine excreted in the urine vary considerably over the course of the day, but the levels vary in parallel. As a result, dividing the amount of DiAcSpm excreted by the amount of creatinine excreted greatly diminishes the variation observed in spot urine samples and yields a good approximation of the total amount of daily DiAcSpm excretion divided by the total amount of daily creatinine excretion. Therefore, any spot urine sample can be used to obtain a reasonable estimate of daily DiAcSpm excretion in nmol DiAcSpm/g creatinine (Hiramatsu et al. 2013).

Although the amount of DiAcSpm in urine from healthy individuals is 30 to 100 times smaller than that of major urinary polyamine components, such as $N^1$- and $N^8$-monoacetylspermidine and monoacetylputrescine, its coefficient of variation (CV) value among 50 persons was comparable to the CV values of major urinary polyamine components. This is remarkable, considering the very low DiAcSpm content, and suggests that DiAcSpm levels in urine vary little from one individual to another. In other words, DiAcSpm is secreted in a highly controlled manner.

Urinary DiAcSpm levels are approximately 1.5 fold higher in females than in males, most likely because creatinine levels are lower in females. Urinary creatinine is primarily derived from creatine and phosphocreatine in muscle tissue, and on average females have lower muscle mass than males. Moreover, in females, urinary DiAcSpm levels vary during the menstrual cycle, increasing before the onset of menstruation and decreasing after the end of menstruation (Hiramatsu et al. 2013).

## 23.3 DiAcSpm as a Tumor Marker

An early study relying on HPLC analysis revealed that total polyamines, acetylputrescine, and $N^8$-acetylspermidine were not significantly increased in a considerable number of cancer patients compared to healthy controls, whereas $N^1$-acetylspermidine was often elevated substantially even in cases of benign diseases. In contrast, the level of urinary DiAcSpm is markedly increased in approximately 80 % of patients with urogenital cancer (Sugimoto et al. 1995; Kawakita and Hiramatsu 2006). Other studies have reported elevated urinary DiAcSpm levels in leukemic patients, as detected by gas chromatographic analysis (van den Berg et al. 1986; Lee et al. 1998). Subsequent analyses using DiAcSpm-specific antibodies indicated that DiAcSpm levels are frequently elevated in patients with various cancers, including colorectal cancer, breast cancer, pancreatobiliary carcinoma, and hepatocellular carcinoma (Kawakita and Hiramatsu 2006). This finding suggests that DiAcSpm is more sensitive than most existing tumor markers (Enjoji et al. 2004; Hiramatsu et al. 2005; Yamaguchi et al. 2005; Umemori et al. 2010; Nakayama et al. 2012). Preliminary studies suggest that DiAcSpm levels are also increased in patients with lung cancer (Sakaguchi et al. 2008; Kato et al. 2011). These examples strongly suggest that DiAcSpm may be useful in detecting a wide variety of neoplastic diseases. It is not unexpected that various cancers are associated with increased DiAcSpm excretion in the urine, as activation of polyamine metabolism is associated with cell proliferation, and polyamine content is increased in rapidly growing cells (Tabor and Tabor 1976).

One of the features that makes urinary DiAcSpm a particularly attractive candidate for use as a tumor marker is that the DiAcSpm level is more frequently elevated in patients in the early stages of colorectal and breast cancer compared to other tumor markers (Hiramatsu et al. 2005; Umemori et al. 2010; Nakayama et al. 2012). For instance, the level of DiAcSpm in the urine is higher than normal in 60 % of early colorectal cancers (i.e., those that are still restricted to the mucosal layers), whereas carcinoembryonic antigen (CEA) is elevated in only 10 % of these patients (Hiramatsu et al. 2005). A tumor marker that facilitates the detection of cancer at early clinical stages is valuable because it enables timely treatment. Importantly, more than 90 % of patients with stage 0 and I colorectal cancer may be cured of disease with proper treatment.

Another advantage of DiAcSpm as a tumor marker is that the quantity of DiAcSpm can be determined from urine samples. Collecting urine is less invasive than obtaining blood samples. All these features suggest that the level of urinary DiAcSpm may be useful as an initial screening tool for cancer.

## 23.4 Simple and Rapid Quantification of DiAcSpm

HPLC analysis (Kabra et al. 1986; Hiramatsu et al. 1994, 1995; Vujcic et al. 2000) was a very useful tool for our early DiAcSpm studies because it enabled us to determine the quantity of multiple urinary polyamines simultaneously and led us to recognize the occurrence of DiAcSpm in healthy human urine (Hiramatsu et al. 1995). Early HPLC analysis further indicated that DiAcSpm, among other urinary polyamines, is particularly promising as a tumor marker, providing a rationale for concentrating on DiAcSpm quantification with the goal of clinical application as a novel tumor marker. For the purpose of clinical applications, however, HPLC and gas chromatography (which was also used in earlier studies) are rather time consuming and inconvenient.

The development of DiAcSpm-specific antibodies was important for constructing simple systems for detecting DiAcSpm in urine. Human urine usually contains 30 times more $N^1$-acetylspermidine than DiAcSpm. Therefore, the cross-reactivity of $N^1$-acetylspermidine with an anti-DiAcSpm antibody should be less than 0.1 % for accurate determination of the quantity of DiAcSpm in urine. Obtaining this highly selective anti-DiAcSpm antibody was not a simple task because the molecular structure of $N^1$-acetylspermidine partially overlaps with DiAcSpm. A sufficiently DiAcSpm-specific antibody was eventually obtained (Hiramatsu et al. 1998), an enzyme-linked immunosorbent assay (ELISA) procedure using this antibody to measure DiAcSpm levels was developed (Hiramatsu et al. 1998), and the use of DiAcSpm as a tumor marker was explored using this and analogous procedures. Subsequently, monoclonal anti-DiAcSpm antibodies with selectivity sufficient for use in urinary DiAcSpm measurements were also developed (Kawakita et al. 2011; Moriya et al. 2014), and an ELISA kit is now commercially available (Umemori et al. 2010; Kawakita et al. 2011).

To facilitate the clinical application of DiAcSpm, it is important to develop and establish a reagent kit that is compatible with the automatic biochemical analyzers that are routinely used in clinical laboratories. We therefore developed a reagent kit based on colloidal gold aggregation (Kawakita et al. 2011). The colloidal gold aggregation method is based on specific binding between a colloidal gold–anti-DiAcSpm antibody complex and a bovine serum albumin (BSA)–acetylspermine conjugate that acts as a DiAcSpm mimic. The DiAcSpm mimic causes aggregation of colloidal gold particles and induces a wine-red to grey color change. DiAcSpm in urine samples competes with the DiAcSpm mimic for binding to the colloidal gold–antibody complex and suppresses the color change in a quantity-dependent manner. This method is more rapid and convenient as compared to the ELISA system. In addition, it is suitable for use in clinical tests because a large number of samples can be processed at one time, and accurate and precise analytical results can be obtained (Samejima et al. 2010).

An immunochromatographic system for simple and convenient DiAcSpm measurement was recently developed for personal use (Moriya et al. 2014). DiAcSpm is more sensitive than most conventional tumor markers in detecting early cancers.

The development of this simple test could prompt apparently healthy individuals to examine their own urine samples for DiAcSpm, encouraging early visits to clinics in the absence of other symptoms and eventually contributing to improving the efficiency of the early detection of cancer. This approach is important in reducing the number of fatal cases of cancer because cancer can often be cured if detected early enough.

## 23.5 Why Does DiAcSpm Indicate the Presence of a Tumor? DiAcSpm Production and Excretion

We do not yet fully understand why elevated levels of urinary polyamines occur in patients with cancer, and why DiAcSpm behaves differently than other polyamine species, making it a useful tumor marker. It is unclear where DiAcSpm is produced and how it is excreted in the urine. Increased production of DiAcSpm in cancer patients may occur in two ways: (1) cancer cells may generate DiAcSpm from intracellular polyamines that are produced at increased levels by actively growing cells, or (2) healthy cells or tissues may in fact be producing increased amounts of DiAcSpm using the increased levels of polyamines in the bloodstream of cancer patients as substrates.

Previous reports indicating an increase of $N^1$-acetylspermidine in colorectal cancer and breast cancer tissues may be consistent with the first alternative (Takenoshita et al. 1984; Kingsnorth and Wallace 1985), although the presence of DiAcSpm in cancer tissues was only demonstrated recently. However, evidence suggesting the involvement of peritoneal macrophages in DiAcSpm production supports the second alternative (Hamaoki and Nagata 2006).

A recent study examined the level of DiAcSpm in colorectal cancer and intraepithelial neoplasia tissues (Kuwata et al. 2013). DiAcSpm was very scant in normal tissues but was markedly increased in resected stage II, III, and IV colon cancer tissues. The level of DiAcSpm was also markedly elevated in metastatic liver lesions in individuals with stage IV colon cancer compared to adjacent normal tissue. HPLC analysis indicated that the levels of $N^1$-acetylspermidine and $N^1$-acetylspermine are also increased in cancer tissues. This is the first report that definitively identifies the presence of DiAcSpm in human tissues. Importantly, DiAcSpm is markedly increased in tumor tissue compared to normal tissue. The levels of $N^8$-acetylspermidine and $N^1$,$N^8$-diacetylspermidine were very low or undetectable in normal tissues and were not significantly increased in cancer tissues.

Tissues from patients with different clinical stages of intraepithelial neoplasia and early colorectal cancer were examined for DiAcSpm, and the tissue DiAcSpm content was compared with normal tissues adjacent to the lesions. The ratio of tumor to normal DiAcSpm content tended to increase from low-grade intraepithelial neoplasias to high-grade neoplasias. The ratio was greater than 1.5 in 78 % of high-grade intraepithelial neoplasia cases examined, indicating that tissue DiAcSpm levels were elevated at very early stages of colorectal cancer development, even before the tissues could be classified as carcinomas on the basis of morphological

criteria (Kuwata et al. 2013). This finding is consistent with the notion that DiAcSpm production in early cancers and precancerous tissues is responsible for the increased excretion of DiAcSpm in the urine of cancer patients, including patients in early clinical stages.

An alternative mechanism involving macrophages has been proposed to explain the increased DiAcSpm production. Peritoneal macrophages from $P388D_1$ lymphoid tumor-bearing mice produced DiAcSpm if exogenous spermine was added as a substrate. In contrast, neither $P388D_1$ cells themselves nor macrophages from healthy mice produced significant levels of DiAcSpm (Hamaoki and Nagata 2006).

It is possible that both cancer cells and macrophages contribute to the increase of urinary DiAcSpm levels in cancer patients. However, it is more likely that cancer tissues are responsible for the increased excretion of DiAcSpm in the urine of early cancer patients, because in the model experiments using $P388D_1$ lymphoid tumor-bearing mice the urinary DiAcSpm levels did not increase until the mice reached the terminal stage of the disease (Hamaoki and Nagata 2006). Notably, urinary DiAcSpm often reaches extraordinarily high levels at the terminal stage of cancers, including colorectal cancer and lymphoma (K. Hiramatsu, unpublished observation).

Whatever the mechanism of DiAcSpm production in tumor-bearing individuals may be, DiAcSpm and other acetylated polyamines are excreted from various tissues and organs into the bloodstream. During circulation, small molecules including polyamines are filtered through the glomerular basement membrane in the kidney. However, a significant portion of the substances in the glomerular filtrate is soon reabsorbed from the brush border of the tubular cells. Polarized pig kidney-derived $LLC\text{-}PK_1$ cells were used as a model for the renal reabsorption system to study the uptake of free and acetylated polyamines from the apical membrane (Miki et al. 2005). The results showed that putrescine, spermidine, and spermine are incorporated into the cells from the apical side via a common transport system. Monoacetylpolyamines also seem to share the same transport system, as monoacetylspermine effectively competed with spermidine for uptake from the apical membrane. Experiments using [$^3$H]-labeled monoacetylspermine confirmed that it entered cells from the apical side. Interestingly, the presence of DiAcSpm did not affect spermidine uptake, indicating that DiAcSpm does not interact with the spermidine transport system. Furthermore, experiments using [$^3$H]-labeled DiAcSpm clearly demonstrated that it was not incorporated at all by these cells.

Monoacetylpolyamines that are reabsorbed by the renal tubular system are converted to free polyamines by acetylpolyamine oxidase (APAO) and subsequently reutilized in the body. Importantly, DiAcSpm is not reabsorbed by this route. It is likely that the total amount of DiAcSpm excreted from cells is recovered in the urine without significant loss, whereas the amount of urinary monoacetylpolyamines decreases by an unknown amount compared to that originally excreted from the cells as a result of renal reabsorption and subsequent reutilization.

Based on these observations, we propose that DiAcSpm production and excretion occur as follows (Fig. 23.1): (1) DiAcSpm is produced in cancer cells, beginning at an early stage of cancer development; (2) DiAcSpm is then excreted from the cancer cells and transported by the bloodstream to the kidney; (3) DiAcSpm appears

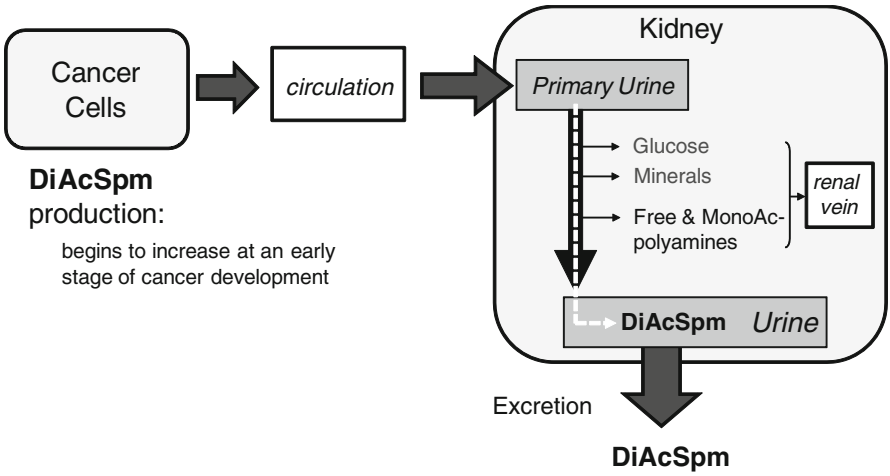


**Fig. 23.1** $N^1$,$N^{12}$-Diacetylspermine (DiAcSpm) production and excretion in the urine. DiAcSpm levels begin to increase in cancer cells in the early stages of cancer development. In the kidney, free polyamines and monoacetylpolyamines in the primary urine are reabsorbed in the renal proximal tubules. DiAcSpm is not reabsorbed and is eventually excreted in the urine

in the glomerular filtrate (primary urine) as well as other polyamines and small molecules such as water, glucose, and minerals; (4) a significant fraction of these small molecules, including free and monoacetylpolyamines, is reabsorbed through the renal tubules and returns to the circulation via the renal vein, whereas DiAcSpm is not reabsorbed; and (5) the total amount of DiAcSpm that occur in the primary urine is excreted in the urine. The process outlined here could explain why the level of urinary DiAcSpm reflects the presence of cancer with high sensitivity and at early clinical stages although the urinary levels of monoacetylpolyamines do not.

Tissue DiAcSpm measurements indicate that DiAcSpm is a characteristic metabolite in malignant tissues. Understanding the mechanism of DiAcSpm production would explain why DiAcSpm serves as a high-performance tumor marker. An important feature of DiAcSpm is that its level begins to increase in tissues at an early stage in cancer development. The increase in DiAcSpm levels or a change in the level of one or more enzymes involved in DiAcSpm metabolism may be a result of malignant transformation. Alternatively, this increase may be more closely related to the mechanism of cancer development. To tackle this challenging question, we need to precisely analyze relevant enzymes at both the gene expression and the enzyme activity level. Increased production of DiAcSpm may be brought about by an increase in spermidine/spermine $N^1$-acetyltransferase (SSAT), or a decrease in APAO, or both. In fact, an increase in SSAT and a decrease in APAO have been reported in breast cancer tissues (Wallace et al. 2000).

However, accurately determining the activity of polyamine catabolizing enzymes is not an easy task. For instance, tissue extracts contain a number of acetyltransferases and their substrates as well as a number of oxidases and their substrates that may

interfere with the determination of SSAT and APAO levels. Recently, we developed a mass spectrometric method to accurately and specifically determine the activities of these enzymes (Moriya et al. 2012). The method consists of determining the quantities of stable isotope-labeled natural polyamine products derived from an appropriately labeled natural polyamine substrate. This method enables us to quantitatively determine the activity of an enzyme with well-defined specificity on a well-defined substrate and clearly distinguish it from background levels caused by contaminants. The method has been successfully used to assess SSAT and APAO activity in crude tissue extracts and will be useful in future studies that aim to elucidate the mechanisms underlying DiAcSpm elevation in tissues early in cancer development.

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# Chapter 24
# Targeting the Polyamine Biosynthetic Pathway in Parasitic Protozoa

**Nahir Velez and Margaret A. Phillips**

**Abstract** Malaria, trypanosomiasis, and leishmaniasis are life-threatening parasitic diseases for which safe and more effective medicines are urgently needed. The polyamine biosynthetic pathway in protozoan pathogens is a validated target for the development of drugs, as demonstrated by the current use of **α**-difluoromethylornithine (DFMO) to treat patients diagnosed with human African trypanosomias (HAT). These parasites have evolved novel polyamine metabolic pathways that are considerably different from those of the human host. For example, trypanosomes contain a novel spermidine/glutathione conjugate termed trypanothione, and uniquely in these parasites *S*-adenosylmethionine decarboxylase (AdoMetDC) is activated by heterodimer formation with a catalytically dead homologue. In *Plasmodium* parasites, AdoMetDC and ODC are fused as a bifunctional protein. *Trypanosoma cruzi* lacks ODC and relies on polyamine transporters. In this chapter, we discuss these differences and highlight additional aspects of these organisms that could be exploited as potential therapeutic strategies, including differences in protein turnover rates and polyamine transport, the latter of which could be used for delivery of cytotoxic compounds.

**Keywords** Chaga's disease • Human African trypanosomiasis (HAT) • Leishmaniasis • Malaria • Ornithine decarboxylase (ODC) • Polyamines • *S*-Adenosylmethionine decarboxylase (AdoMetDC) • Spermidine synthase (SpdSyn) • **α**-Difluoromethylornithine (DFMO)

## 24.1 Introduction

Protozoan pathogens represent a potential threat for a vast number of people worldwide. They are causative agents of some of the most detrimental parasitic diseases, such as malaria, human African trypanosomiasis (HAT), leishmaniasis, Chagas

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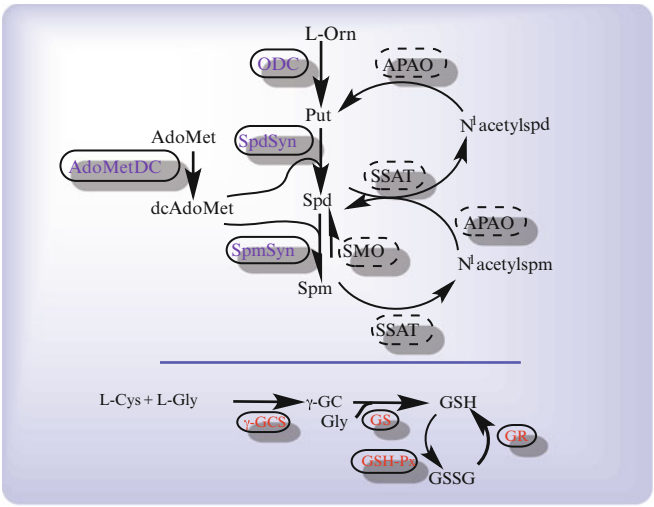
disease, and toxoplasmosis, among others. The burden of these diseases primarily impacts subtropical and tropical regions of developing countries in which economy, nutrition, and public health are major concerns. For the trypanosomatids, current drugs are far from ideal because of high cost, poor compliance, difficult administration regimes, drug resistance, and safety issues (Stuart et al. 2008; Jacobs et al. 2011; Barrett and Croft 2012). For malaria, although many effective therapies have been developed, the continuing problem of drug resistance leads to a need for a robust pipeline of new drug discovery (Miller et al. 2013; Burrows et al. 2011, 2013). Fortunately, recent attempts have been made to support and facilitate discovery and development of new drugs against these deadly diseases, including publicly funded whole genome sequencing of protozoan parasite genomes, establishment of public-private partnerships that support drug discovery [e.g., Medicines for Malaria Venture (MMV) and Drugs for Neglected Diseases initiative (DNDi)] and availability of funds from scientific research by organizations such as the National Institutes of Health (NIH) and the Bill and Melinda Gates Foundation. Based on these opportunities, different strategies are being used to produce safer and more effective therapies, which can be classified as short- or medium-term (e.g., modification of current drugs) or long-term (e.g., discovery of new drugs) approaches. The latter, in particular, often begins with the identification of molecular targets through genetic and biochemical techniques. Most common drug targets work by influencing cellular processes such as gene transcription/translation, metabolism, signal transduction, protein turnover, transport, redox mechanisms, and immune responses. Polyamine biosynthesis is a key process that has been extensively studied as a potential target for treating infectious and proliferative diseases (Casero and Pegg 2009; Gerner and Meyskens 2004; Jacobs et al. 2011).

Polyamines are commonly associated with several cancer malignancies. They increase cell proliferation, decrease apoptosis, and increase expression of genes involved in tumor invasion and metastasis (Casero and Pegg 2009; Gerner and Meyskens 2004). Eflornithine (α-difluoromethylornithine or DFMO), which irreversibly inactivates ornithine decarboxylase (ODC), is the most widely studied example of a polyamine metabolism inhibitor that suppresses cancer development in animal models and has been evaluated in clinical trials (Casero and Pegg 2009; Gerner and Meyskens 2004). However, in addition to cancer, polyamines have also shown to play crucial roles in parasitic diseases. In fact, the only clinically approved use of a polyamine biosynthetic inhibitor for treatment of a systemic illness is for the treatment of HAT (also known as African sleeping sickness), for which the mechanism of action has been fully validated (Jacobs et al. 2011). This finding demonstrates that the polyamine biosynthetic pathway represents an important tool that can be exploited for the development of newer and safer antiparasitic therapies. The polyamine metabolic pathway is distinct in many of the protozoal parasites, providing potential mechanisms for species-selective targeting (Birkholtz et al. 2011; Willert and Phillips 2012). The unique aspects of the polyamine biosynthetic pathway in the protozoal parasites are discussed next.

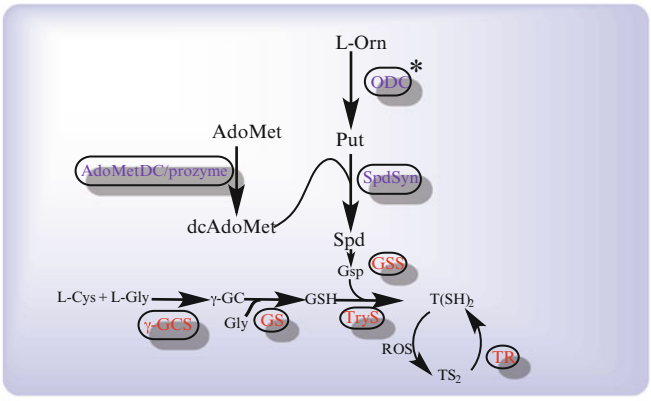
### 24.1.1 Polyamine Biosynthetic Pathway in the Trypanosomatids

*Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* spp. belong to a specific group of single-cell flagellate protozoa known as trypanosomatids (Stuart et al. 2008). The human pathologies associated with these species are HAT, American trypanosomiasis (also known as Chagas disease), and leishmaniasis, respectively. Trypanosomatids have evolved a number of novel aspects of their polyamine biosynthetic pathways (Willert and Phillips 2012). Key differences between the parasite and the human host pathways are depicted on Fig. 24.1. Trypanosomatids do not contain the polyamine spermine, which is formed from spermidine in mammalian cells by spermine synthase (SpmSyn). Instead, spermidine is conjugated to glutathione by glutathionylspermidine synthetase (GSS) and trypanothione synthetase (TryS) to form a novel cofactor, termed trypanothione, which is essential for the parasites to avoid oxidative stress and maintain a thiol redox balance. In humans, gluthatione reductase (GR) is the key enzyme required to control oxidative stress by producing reduced glutathione, which scavenges reactive oxygen species. However, in the trypanosomatids, trypanothione reductase (TR) replaces the function of GR (Krauth-Siegel et al. 2007). In addition, the genomes of the trypanosomatids lack the catabolic enzymes, polyamine oxidase (APAO) and spermidine/spermine $N^1$-*acetyltransferase* (SSAT), required for converting the longer-chain polyamines back to putrescine. The catabolic enzymes are important components of the polyamine regulatory mechanism in mammalian cells. Finally, we discovered that the structure and regulation of AdoMetDC is novel in these parasites. AdoMetDC is active as a homodimer in mammalian cells; however, in the trypanosmatids the active AdoMetDC is a heterodimer formed by an impaired but active subunit and a catalytically dead paralogue of the active AdoMetDC, named Prozyme (Willert et al. 2007; Willert and Phillips 2009). Prozyme lacks key catalytic residues and is not processed to form the pyruvoyl group. Heterodimer formation between the active AdoMetDC paralogue and prozyme leads to an increase in enzyme catalytic efficiency ($k_{cat}/K_m$) of three orders of magnitude. The heterodimeric species was also shown to be the functional form of the enzyme in the parasite (Willert and Phillips 2008), and AdoMetDC prozyme was shown to have a likely regulatory role in polyamine homeostasis (discussed below) (Willert and Phillips 2008; Xiao et al. 2013). Interestingly, we found this activation mechanism was also required for formation of the active and functional trypanosomatid deoxyhypusine synthase (DHS), which catalyzes the spermidine-dependent modification of the translation initiation factor eIF5A (Nguyen et al. 2013). Trypanosomes lack many of the classic regulatory mechanisms for gene expression that are found in mammalian cells, including the mechanisms for regulation of polyamine metabolic enzymes (described in other chapters in this book). That this novel regulatory mechanism has evolved independently twice within the polyamine pathway in these parasites suggests that there is a strong drive to regulate polyamine biosynthesis and utilization, and thus in the absence of other mechanisms the parasites have relied on the use of pseudoenzymes to regulate protein function.

*Humans*




*Trypanosomatids*




*ODC not present in *T. cruzi* and putrescine is imported from the host cell.

*Plasmodium*

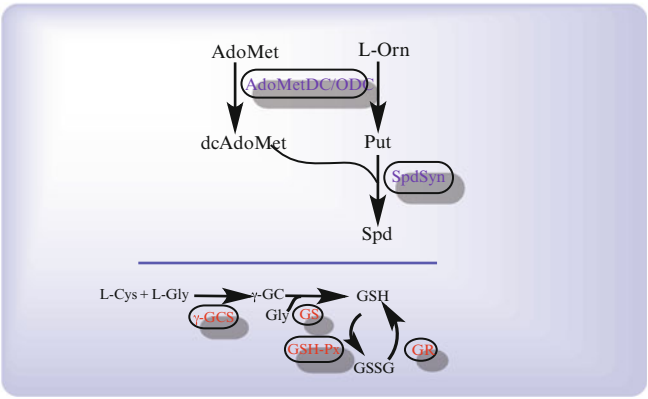



**Fig. 24.1** Polyamine and glutathione metabolic pathway differences in parasite and mammalian cell enzymes involved in polyamine biosynthesis (*purple* font inside *solid ovals*), catabolic enzymes (*black* font inside *dashed ovals*), and enzymes involved in glutathione metabolism (*red* font inside *solid ovals*). *ODC* ornithine decarboxylase, *AdoMetDC* *S*-adenosylmethionine decarboxylase, *SpdSyn* spermidine synthase, *SpmSyn* spermine synthase, *SMO* spermine oxidase, *SSAT* spermidine/spermine $N^1$ acetyltransferase, *APAO* $N^1$ acetyl polyamine oxidase, *γGCS*

### 24.1.2 *Human African Trypanosomiasis (HAT, Also Known as Sleeping Sickness)*

Sleeping sickness is most commonly found in the poorest regions of sub-Saharan Africa, covering 36 countries in which about 50 million people are estimated to be at risk (Kennedy 2013). *Trypanosoma brucei* avoids the immune response by undergoing extensive antigenic variation of a surface protein named variant surface glycoprotein (VSG) (Rudenko 2011). This situation makes developing a vaccine a very challenging task. Currently used drugs for treating HAT patients display significant toxicity issues and adverse reactions to extensive administration regimes (Jacobs et al. 2011; Barrett and Croft 2012). DFMO is the most recently discovered drug developed for treating late-stage disease and has significant safety advantages over Melarsoprol (Balasegaram et al. 2009). In a landmark study by Bacchi and coworkers (Bacchi et al. 1980) it was initially found to have anti-trypanosomal activity, and it was registered for treatment of West African sleeping sickness caused by *T. brucei gambiense* infections in 1990. It was found to be less effective against *T. brucei rhodesiense* (Bacchi et al. 1990). Additionally, as a single agent DFMO had to be administered in high doses (400 mg/kg daily given as 56 intravenous infusions over 14 days), which made administration difficult in rural Africa (Balasegaram et al. 2009). In an effort to develop a more efficacious drug, nifurtimox, which is used for treating Chagas disease, was tested in combination with DFMO and found to shorten the treatment course (Priotto et al. 2009; Yun et al. 2010). The combination therapy, named NECT (15 mg/kg oral nifurtimox for 10 days and 400 mg/kg eflornithine in 14 i.v. infusions over 7 days), was both safer and more efficacious than DFMO alone. However, NECT is still not approved for treatment of *T. brucei rhodesiense* infections because of the reduced activity of DFMO on this parasite. Although DFMO resistance has not been demonstrated clinically, generation of resistant cell lines in the laboratory has been possible, and in a seminal recent study Barrett and coworkers characterized DFMO-resistant cell lines identifying the DFMO transporter in *T. brucei* (Vincent et al. 2010).

#### 24.1.2.1 *Ornithine Decarboxylase* (ODC)

Genetic and chemical studies in *T. brucei* have demonstrated that the polyamine and trypanothione biosynthetic enzymes are essential for growth and survival of these parasites (Willert and Phillips 2012). *T. brucei* strains that lack both wild-type *ODC*

γ-glutamylcysteine synthetase, *GS* glutathione synthetase, *GR* glutathione reductase, *GSS* glutathionylspermidine synthetase, *TryS* trypanothione synthetase, *TR* trypanothione reductase, *GSH-Px* glutathione peroxidase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *Orn* ornithine, *Put* putrescine, *Spd* spermidine, *Spm* spermine, *AdoMet S*-adenosylmethionine, *dcAdoMet* decarboxylated *S*-adenosylmethionine, l-*Cys* L-cysteine, l-*Gly* L-glycine, *γGC* γ-glutamylcysteine, *GSH* glutathione, *Gsp* glutathionylspermidine, $T(SH)_2$ reduced trypanothione, $TS_2$ oxidized trypanothione

alleles are putrescine auxotrophs when grown in vitro (Li et al. 1998). Similar results were also obtained by RNA interference (RNAi) (Xiao et al. 2009). Notably, polyamines are found in very low quantities in the plasma of the mammalian host and the polyamine transport system of these organisms is inefficient (Heby et al. 2003). Thus, there is no possibility for the inhibition of *T. brucei* ODC to be abolished in vivo. In fact, when *T. brucei* deficient in ODC were used to infect mice, parasites were unable to multiply and were rapidly cleared from the blood (Li et al. 1998). Crystal structures of both human and *T. brucei* ODC have been solved and show that the active site of these enzymes, including the DFMO-binding site, are quite similar (Grishin et al. 1999; Almrud et al. 2000). However, the intracellular turnover rate of human ODC ($t_{1/2} < 1$ h) is considerably faster than that of the stable trypanosomatid enzyme, a difference that is thought to lead to selective inhibition of the parasite enzyme over the host (Phillips et al. 1987). Attempts to identify reversible *T. brucei* ODC inhibitors that might overcome some of the poor pharmacology associated with DFMO were undertaken, including a larger high throughput (HTS) chemical screen (Smithson et al. 2010a, b). The results from this study were disappointing in that only a handful of novel scaffolds were identified. The data suggested that ODC is a poor target for the identification of compounds that are more typically drug like and that function through reversible inhibition.

#### 24.1.2.2 *S*-Adenosylmethionine Decarboxylase (AdoMetDC)

Knockdown of AdoMetDC by RNAi in the *T. brucei* bloodstream from parasites demonstrated the essentiality of *T. brucei* AdoMetDC, and generation of a regulated knockout construct showed that the regulatory subunit prozyme was also essential (Willert and Phillips 2008). AdoMetDC inhibitors were shown to cure *T. brucei* infections in mice: these include MDL 73811, a suicide inhibitor, some MDL 73811 analogues, and several aryl and heteroaryl bis-(guanylhydrazones), which are potent reversible inhibitors of the enzyme (Bitonti et al. 1990; Bacchi et al. 1992, 1996; Barker et al. 2009; Hirth et al. 2009). Unfortunately, most of these compounds displayed significant pharmacokinetic issues and could not cross the blood–brain barrier, impeding progression through the drug discovery pipeline. However, these compounds still served to chemically validate AdoMetDC as a target.

Regulation of polyamine levels in trypanosome parasites was not well understood until the finding that *T. brucei* AdoMetDC is activated by heterodimer formation with a catalytically dead homologue named prozyme, (Willert et al. 2007; Willert and Phillips 2008). Not only did prozyme activate AdoMetDC upon heterodimer formation, but prozyme protein levels were found to respond to treatments that depleted AdoMetDC activity (Willert and Phillips 2008; Xiao et al. 2013). Both knockdown of AdoMetDC by RNAi or the chemical inhibition by AdoMetDC inhibitors led to a 10- to 20-fold upregulation of prozyme protein levels. Metabolite analysis showed that, in contrast to in mammalian cells, spermidine was not responsible for regulating expression levels of prozyme, and instead the data suggested that decreasing levels of decarboxylated AdoMet (dcAdoMet) may be the regulatory

trigger for this response. Crystallographic information is available for human AdoMetDC, but not for the trypanosomal enzyme; however, our recent site-directed mutagenesis studies showed that key residues (Leu-8, Leu-10, Met-11, Met-13) at the N-terminus of *T. brucei* AdoMetDC are essential for allosteric activation by the prozyme (Velez et al. 2013). These data suggest that compounds designed to disrupt the AdoMetDC–prozyme complex interface, to target the N-terminal region, or to lock the protein in the inactive conformation could all be successful approaches.

#### 24.1.2.3 Spermidine Synthase (SpdSyn)

Knockdown of SpdSyn by RNAi in the bloodstream from *T. brucei* parasites also led to growth arrest (Xiao et al. 2009; Taylor et al. 2008). The growth arrest could be rescued by spermidine, but these studies and others (Willert and Phillips 2008) have shown that spermidine is not transported efficiently in these parasites. Unfortunately, the most potent SpdSyn inhibitor, dicyclohexylamine, did not increase the survival time of mice infected with trypanosomes, and thus discovery of new compounds is necessary (Birkholtz et al. 2011).

#### 24.1.2.4 Trypanothione Synthase (TryS) and Trypanothione Reductase (TR)

Enzymes involved in polyamine-dependent redox metabolism have also been genetically validated as essential targets in *T. brucei* (Huynh et al. 2003; Krieger et al. 2000; Ariyanayagam et al. 2005; Wyllie et al. 2009; Torrie et al. 2009). Although numerous reports of TR inhibitors have been described identifying inhibitors with anti-trypanosomal activity, clear chemical validation of the target has been elusive (e.g., Patterson et al. 2011). In contrast, inhibitors of TS have been shown to be on target in their parasite-killing activity, validating this target for future drug discovery efforts (Wyllie et al. 2009; Torrie et al. 2009; Spinks et al. 2012).

## 24.2 American Trypanosomiasis (Also Known as Chagas Disease)

Chagas disease, another form of trypanosomiasis, is common in Central and South America and affects about 8 to 10 million people living in the endemic regions (Nunes et al. 2013). It is caused by the protozoan pathogen *Trypanosoma cruzi*. The currently available drugs to treat Chagas disease are benznidazole and nifurtimox, but none of them is effective for treating patients in the chronic stage. An important difference in polyamine biosynthesis within the trypanosomatids is that while *T. brucei* and *Leishmania* spp. are able to synthesize putrescine de novo, *T. cruzi* lacks ODC and instead salvages it from the host cell, where polyamines are plentiful

(Birkholtz et al. 2011; Heby et al. 2007) (Fig. 24.1). Although *T. cruzi* also lacks arginase, which produces ornithine, genes for SpdSyn and AdoMetDC are present in the parasite to enable the conversion of the imported putrescine to spermidine. *T. cruzi* AdoMetDC is also activated by heterodimer formation with the respective prozyme (Willert and Phillips 2009). In this particular case, putrescine is necessary for the heterodimeric enzyme to reach a fully active state. This requirement is not observed in *T. brucei* and might be related to the lack of ODC in *T. cruzi*. The lack of ODC makes treatment of Chagas disease patients with DFMO an unfeasible approach. Additionally, inhibitors of human AdoMetDC, such as methylglyoxal-bis(guanylhydrazone) (MGBG) and other derivatives, were tested in vitro against the *T. cruzi* enzyme and had a poor effect (Brun et al. 1996). MDL 73811, the suicide inhibitor of *T. brucei* AdoMetDC, showed better efficacy when evaluated in a rat heart myoblast infection model (Yakubu et al. 1993). However, because exogenous spermidine did not reverse the effect on parasite infectivity, it was hypothesized that the outcome was a consequence of AdoMet accumulation rather than polyamine depletion. Blocking the polyamine transporter, which is highly efficient, or utilizing this transport mechanism to deliver cytotoxic compounds might represent an alternative and more fruitful avenue for targeting the polyamine pathway in *T. cruzi* (Hasne et al. 2010; Hasne and Ullman 2011). The crystal structure of *T. cruzi* SpdSyn has been solved, but inhibitor studies on this enzyme are limited (Fan et al. 2008). It is important to note that formation of trypanothione is catalyzed by a single enzyme (Oza et al. 2002). Thus, because deficiency in putrescine and spermidine can be compensated through the polyamine transport system, targeting TryS might be a more direct and efficient method for the development of new therapies.

## 24.3 Leishmaniasis

Leishmaniasis is one of the major insect-borne diseases in developing countries and affects about 350 million people (Barrett and Croft 2012; Stuart et al. 2008; Croft et al. 2006). *Leishmania* causes a spectrum of diseases, normally divided into cutaneous (CL), the most common form, mucocutaneous (MCL), and visceral leishmaniasis (VL), which is the most severe form that affects essential organs and can be lethal if untreated. Drug treatments for VL include pentavalent antimonials, amphotericin B, paromomycin, and miltefosine; however, clinical efficacy varies with a range of contributing factors including overall patient health, geographic region, coinfections, and drug resistance (Monge-Maillo and Lopez-Velez 2013). The polyamine biosynthetic pathway of *Leishmania* spp. is similar to *T. brucei* (Fig. 24.1). *Leishmania* species also produce a catalytically dead AdoMetDC homologue designated prozyme, although this heterodimer has not yet been biochemically characterized (Willert et al. 2007). Genetic depletion of ODC, AdoMetDC, SpdSyn, and arginase in *L. donovani* promastigotes showed they were required for parasite viability and proliferation (Gilroy et al. 2011b; Roberts et al. 2001, 2002, 2004,

2007; Gaur et al. 2007), and several of the enzymes including ODC have been shown to be necessary for virulence in mice (Boitz et al. 2009) and SpdSyn (Gilroy et al. 2011a). The arginase crystal structure has recently been reported, which would benefit any drug discovery efforts against this target (D'Antonio et al. 2013). DFMO has shown some activity against *Leishmania* species as has the AdoMetDC inhibitor MDL 73811 (Birkholtz et al. 2011). It should be noted that *Leishmania* parasites are also able to import polyamines from their environment, which could influence the inhibitory effect of compounds targeting the pathway (Hasne and Ullman 2011; Landfear 2011).

## 24.4 Malaria

Malaria is considered by the World Health Organization (WHO) to be one of the most serious, life-threatening parasitic diseases in the world. It affects about 500 million people, particularly in sub-Saharan Africa and South Asia, and kills an estimated 0.6 million persons each year (Burrows et al. 2011, 2013; Miller et al. 2013). The disease is caused by protozoan pathogens of the genus *Plasmodium*, which are transmitted to humans by the *Anopheles* mosquito. Traditionally, disease treatment relied on a group of diverse antimalarial compounds that included chloroquine or related compounds (e.g., mefloquine) and antifolates (e.g., pyrimethamine and sulfadoxine). The loss of these compounds to drug resistance, and concerns about the vulnerability of single agents to be lost to resistance, led to the development of new treatments such as the artemisinin-based combination therapies (ACTs). Although ACTs are effective and well tolerated by patients, they too are now threatened by potential drug resistance that appears to be emerging in the field (Fairhurst et al. 2012; Miotto et al. 2013). The polyamine and glutathione biosynthetic pathways are separate in *Plasmodium* parasites (Birkholtz et al. 2011; Clark et al. 2010) (Fig. 24.1). However, a significant difference between *Plasmodium falciparum* and mammalian cells is that in these parasites ODC and AdoMetDC form a bifunctional protein. The catalytically active AdoMetDC forms the N-terminus, which is connected to the C-terminal ODC region through a hinge domain. The enzyme dimerizes through the ODC domain. Structural information for the mature heterotetrameric complex is not available. The significance of the bifunctional enzyme is still not well understood, but it is postulated to play a role in regulation of polyamine pools. Another significant difference between the malaria parasite and human cells is found in the salvage pathway that recycles the polyamine byproduct 5′-methylthioadenosine back to the adenine and methionine pools. Both adenosine deaminase and purine nucleoside phosphorylase have unusual substrate specificity that allows them to be used as dual-purpose enzymes in the recycling of methythiopurine and for purine salvage (Ting et al. 2005). High affinity transition state inhibitors of these enzymes have been reported (Cassera et al. 2011). The efficacy of ODC inhibitors for treating malaria depends on the specific stage of the *Plasmodium* life cycle (Bitonti et al. 1987). DFMO inhibited growth of the asexual stage, the infected red blood cell stage

of *P. falciparum*. Other ODC inhibitors have also been tested in vitro, displaying higher efficiency than DFMO, but growth inhibition was reversed by putrescine (Das Gupta et al. 2005). DFMO did not extend survival time of *Plasmodiuim berghei*-infected mice, suggesting that polyamine uptake from the erythrocyte is sufficient to support growth. Indeed *P. falciparum* has been shown to have robust transporters for both putrescine and spermidine (Niemand et al. 2012). This finding implies that, similar to *T. cruzi*, targeting the polyamine uptake mechanism in *P. falciparum* could be a good approach for treatment. AdoMetDC inhibition in the bifunctional protein with MDL 73811 in *P. falciparum* results in growth arrest in vitro but had no effect in animal models (Wright et al. 1991a, b). Coinhibition of the enzymatic complex with both DFMO and MDL 73811 in vitro resulted in additive growth arrest and complete polyamine depletion, but the data suggested that changes in transcript levels might limit effectiveness (van Brummelen et al. 2009). SpdSyn has not been as thoroughly studied as AdoMetDC and ODC, but inhibition with CHA, 4MCHA, and dicyclohexylamine displayed a noticeable reduction in *P. falciparum* cell growth in vitro (Birkholtz et al. 2011). Polyamine analogs have been reported to have potent activity against *P. falciparum*, although they were only marginally effective in vivo and the mechanism of action of these compounds is unclear (Liew et al. 2013). Overall, the data suggest that inhibition of a single polyamine biosynthetic enzyme will be unlikely to lead to effective parasitological cures of malaria in vivo. Thus, if polyamine biosynthesis is to be effectively targeted in this parasite it likely will require targeting both biosynthesis and uptake.

## 24.5 Conclusions

Polyamine biosynthesis in protozoan pathogens is a well-known target for the development of new therapies against tropical neglected diseases, as demonstrated by the effectiveness of DFMO for treating human African trypanosomiasis (HAT) patients. Key enzymes involve in the process (ODC, AdoMetDC, and SpdSyn) have demonstrated to be essential for parasite proliferation and survival. The particular case of *T. cruzi* parasites, which lack ODC, can be evaluated by blocking the very efficient polyamine transport system these species have or the enzymes involved in polyamine-dependent redox metabolism. A similar approach can be taken against *Plasmodium* parasites, which contain a characteristic AdoMetDC-ODC bifunctional enzyme but also have robust transport mechanisms. The potential to exploit polyamine biosynthesis as a target in the parasitic protozoa remains strongest for treatment of HAT, which is caused by the extracellular *T. brucei* parasite. The recent discovery that trypanosomatid AdoMetDC activity is controlled by the inactive homologue prozyme represents a unique alternative for further exploitation of this enzyme as a drug target and can be utilized as a means for selectively interfering with parasite survival in the human host. Structural information on AdoMetDC-prozyme would be extremely beneficial for rational inhibitor design. Modification of current drugs and targeted drug delivery are crucial strategies that can also lead to better antiparasitic therapeutics.

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# Index





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